mTOR KINASE-ASSOCIATED PROTEINS

RELATED APPLICATIONS

This application claims the benefit of priority of U.S. Provisional Application number 60/448,035 filed February 18, 2003. The entire teachings of the referenced Provisional Application are incorporated herein by reference in its entirety.

FUNDING

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BACKGROUND OF THE INVENTION

Cell growth is the fundamental biological process whereby cells accumulate mass and is an important determinant of the sizes of cells, organs, and organisms (Conlon, I. and Raff, M. (1999), Cell *96*, 235-44; Dixon, D. and Fordham-Skelton, T. (1998), Curr. Opin. Plant Biol. *1*, 1; Gomer, R. H. (2001), Nat. Rev. Mol. Cell Biol. *2*, 48-54; Johnston, L. A. and Gallant, P. (2002), Bioessays *24*, 54-64; Stocker, H. and Hafen, E. (2000), Curr. Opin. Genet. Dev. *10*, 529-35). The mTOR pathway, along with the PI-3Kinase/PKB/PTEN axis, is emerging as a critical regulator of growth in mammals in response to nutrients, hormones and growth factors (Gingras, A. C., et al., (2001), Genes Dev. *15*, 807-26; Kozma, S. C. and Thomas, G. (2002), Bioessays *24*, 65-71; Schmelzle, T. and Hall M. N. (2000), Cell *103*, 253-62). The central component of the pathway, mTOR (also known as RAFT1 or FRAP), was discovered during studies into the mechanism of action of rapamycin (Brown, E. J., et al. (1994), Nature *369*, 756-758; Sabatini, D. M., et al. (1994), Cell *78*, 35-43; Sabers, C. J., et al. (1995), J. Biol. Chem. *270*, 815-822), an anti-proliferative drug with valuable immunosuppressive and anti-cancer clinical applications (Saunders, R. N., et al. (2001), Kidney Int. *59*, 3-16; Vogt, P. K. (2001), Trends Mol. Med. 7, 482-4). mTOR is a member of the PIK-related family of large protein kinases (Keith, C. T. and Schreiber, S. L. (1995), Science *270*, 50-1) and mediates the phosphorylation of at least two regulators of protein synthesis and cell growth: S6 Kinase 1 (S6K1) and an inhibitor of translation initiation, the eIF-4E binding

protein 1 (4E-BP1) (Brunn, G. J., et al. (1997), Science *277*, 99-101; Burnett, P. E., et al. (1998), PNAS *95*, 1432-1437; Isotani, S., et al. (1999), J. Biol. Chem. *274*, 34493-8). Recent work suggests that deregulation of the mTOR pathway plays a role in the pathogenesis of human disease, as the pathway is constitutively active in tuberous sclerosis (Goncharova, E. A. (2002), J. Biol. Chem. *277*, 30958-67; Kwiatkowski, D. J., et al. (2002), Hum. Mol. Genset *11*, 525-34), a tumor-prone syndrome caused by mutations in the TSC1 (van Slegtenhorst, M., et al. (1997), Science *277*, 805-8) or TSC2 (Consortium, T .E. C. T. S. (1993), Cell *75*, 1305-15) genes. Exactly how the mTOR, TSC1/2 and PI-3K/Akt/PTEN pathways interconnect is unknown, but it is likely that these systems integrate growth factor- and nutrient-derived signals to determine overall growth rates. The mTOR pathway is particularly sensitive to the levels of nutrients, such as amino acids (Hara, K., et al. (1998), J. Biol. Chem. *273*, 14484-94) and glucose (Dennis, P. B., et al. (2001), Science *294*, 1102-5; Kim, D. H., et al. (2002), Cell *110*, 163-75), but the molecular mechanisms by which nutrients regulate mTOR are to be understood.

It would be helpful to have methods and compositions that regulate the mTOR pathway, which would be useful as therapeutic approaches for diseases such as cancer, diabetes, and cardiovascular diseases.

SUMMARY OF THE INVENTION

The present invention relates to the discovery of two mTOR-associated proteins (mTOR-APs), termed GβL (G protein β-subunit-like protein) and rictor (rapamycin insensitive companion of mTOR). GβL and rictor are screening targets for the identification and development of novel pharmaceutical agents which modulate the activity of these mTOR-APs. Activities of the mTOR-AP include, but are not limited to, forming a complex with an mTOR protein, activation of the mTOR kinase, modulating signaling through the mTOR pathway, stimulating phosphorylation of PKCα, modulating the actin cytoskeleton organization, and inhibiting or reducing cell growth or proliferation.

In certain embodiments, the invention provides isolated mTOR-AP (e.g., GβL and rictor) polypeptides. Polypeptide fragments or variants of an mTOR-AP polypeptide are

additional embodiments of this invention. The invention additionally relates to isolated nucleic acids (e.g., DNA, RNA) encoding an mTOR-AP polypeptide, mTOR-AP fragments, and mTOR-AP variants. The invention further relates to nucleic acids that are complementary to nucleic acid encoding an mTOR-AP polypeptide. In certain embodiments, the invention relates to nucleic acid which hybridizes under high stringency conditions to all or a portion of nucleic acid encoding an mTOR-AP polypeptide.

In certain embodiments, the invention provides expression vectors comprising nucleic acid encoding an mTOR-AP polypeptide, such as a GβL polypeptide and a rictor polypeptide. Host cells comprising exogenous nucleic acid (e.g., DNA, RNA) encoding an mTOR-AP polypeptide, such as host cells containing an expression vector comprising nucleic acid encoding an mTOR-AP polypeptide, are also the subject of this invention.

In certain embodiments, the invention relates to a method for producing an mTOR-AP polypeptide, such as a method of producing a GβL polypeptide or a rictor polypeptide in isolated host cells containing a vector expressing a GβL or a rictor polypeptide. In certain aspects, the invention relates to an antibody that is specific for a GβL polypeptide or a rictor polypeptide of the invention.

In certain embodiments, the invention provides a method for detecting the presence of the subject mTOR-AP polypeptide (e.g., GβL or rictor) in a sample. This method comprises: a) contacting the sample with an antibody which selectively binds to the mTOR-AP polypeptide; and b) determining whether the antibody binds to the mTOR-AP polypeptide in the sample. In another embodiment, the invention provides a kit for detecting an mTOR-AP polypeptide. The kit comprises an antibody of the invention and a detectable label for detecting said antibody.

In certain embodiments, the invention provides a method for detecting the presence of the subject mTOR-AP nucleic acid (e.g., GβL or rictor) in a sample. This method comprises: a) contacting the sample with an mTOR-AP probe or primer; and b) determining whether the probe or primer binds to the mTOR-AP nucleic acid in the sample. In another embodiment, the invention provides a kit for detecting an mTOR-AP nucleic acid. The kit comprises an mTOR-AP nucleic acid as a probe or a primer and instructions for use.

In certain embodiments, the invention provides an isolated, purified or recombinant complex comprising an mTOR polypeptide and an mTOR-associated protein (mTOR-AP). In one specific embodiment, the complex of the invention comprises an mTOR polypeptide and a GβL polypeptide. In this specific embodiment, the complex further comprises a raptor polypeptide in addition to the mTOR polypeptide and the GβL polypeptide. In another specific embodiment, the complex of the invention comprises an mTOR polypeptide and a rictor polypeptide. In this specific embodiment, the complex further comprises a GβL polypeptide in addition to the mTOR polypeptide and the rictor polypeptide.

In certain embodiments, the invention provides a method of screening for compounds which modulate the activity or expression of an mTOR-AP, such as a GβL protein or a rictor protein. Compounds (e.g., agonists or antagonists) which modulate the mTOR-AP activity or expression are also the subject of this invention.

In certain embodiments, the invention provides a method of inhibiting aberrant activity of an mTOR-AP (e.g., a GβL protein or a rictor protein) in a cell. In this method, a cell is contacted with a compound that modulates the activity or expression of the mTOR-AP, in an amount which is effective to reduce or inhibit the aberrant activity of the mTOR-AP. An exemplary compound includes, but is not limited to, a peptide, a phosphopeptide, a small organic molecule, an antibody, and a peptidomimetic. In this method, a specific cell is a cancer cell. A preferred cell is a human cell.

In certain embodiments, the invention provides a method of treatment for a disease (disorder or condition) affected by aberrant activity of an mTOR-AP (e.g., GβL or rictor). Such disease (disorder or condition) is responsive to mTOR-AP modulation. In this method, a compound that modulates the mTOR-AP activity or expression is administrated to an individual (subject or patient) in need thereof, in a therapeutically effective amount, such that the aberrant activity or expression of the mTOR-AP is reduced or inhibited. Examples of such diseases include, but are not limited to, cancer, diabetes, and cardiovascular diseases (e.g., restenosis).

In another embodiment, the invention provides a transgenic mouse having germline and somatic cells comprising a chromosomally incorporated transgene that disrupts the genomic mTOR-AP gene (e.g., GβL or rictor) and inhibits expression of said

gene. In certain cases, the mTOR-AP gene is disrupted by insertion of a selectable marker sequence. Optionally, the transgenic mouse of the invention exhibits increased or decreased susceptibility to the formation of tumors as compared to the wildtype mouse. The transgenic mouse can be homozygous r heterozygous for the disruption.

In other embodiments, the invention relates to use of a GβL polypeptide or a rictor polypeptide, a nucleic acid encoding a GβL or a rictor polypeptide, or an antibody specific for a GβL or a rictor polypeptide, and a compound which modulates GβL or rictor activity in the manufacture of a medicament for the treatment of diseases affected by GβL and/or rictor activity (e.g., cancer, diabetes, or cardiovascular disorders). GβL and/or rictor nucleic acids and the proteins encoded thereby, as well as the fragments and variants thereof, can be used as therapeutic drugs, drug targets, and for diagnostic purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1E show identification of GβL as an mTOR-associated protein. (A) Coomassie blue stained SDS-PAGE analysis of mTOR immunoprecipitates prepared from HEK-293T cell extracts in the absence or presence (+pep) of the blocking peptide for the anti-mTOR antibody. Quantitation by densitometry (Kim et al., 2002) of the bands corresponding to mTOR and p36 reveals a ratio of 1.0 mTOR to 0.9 p36. (B) Specific in vivo interaction between mTOR and GβL. A polyclonal anti-GβL antibody recognizes GβL in mTOR immunoprecipitates prepared from cells lysed in buffers containing either 0.3% CHAPS or 1% Triton X-100 but not in immunoprecipitates prepared with the indicated control antibodies or in the presence of the blocking peptide for the mTOR antibody. (C) Endogenous mTOR interacts with recombinant GβL but not γ-tubulin. Immunoblotting for mTOR and myc-tagged proteins was performed on anti-myc immunoprecipitates prepared from HEK-293T cells expressing myc-GβL or myc-γ-tubulin. (D) Recombinant versions of mTOR and GβL interact with each other. Immunoblotting of HA-mTOR and myc-tagged proteins was performed on myc immunoprecipitates prepared from HEK-293T cells coexpressing HA-mTOR with either myc-GβL or myc-γ-tubulin. (E) GβL interacts with the mTOR kinase domain. Myc-tagged full-length mTOR, its indicated fragments or γ-tubulin were co-expressed with

HA-GβL, and anti-myc immunoprecipitates analyzed by anti-HA and anti-myc immunoblotting.

Figures 2A-2E show that GβL has a positive function in the mTOR pathway. (A) Inhibition of S6K1 phosphorylation in cells transfected with siRNAs targeting GβL. Cell lysates prepared from HEK-293T cells transfected with the lamin, mTOR, or GβL siRNAs were analyzed with immunoblotting for the indicated proteins. (B) Transfection of the siRNA targeting GβL eliminates S6 phosphorylation. HeLa cells transfected with the indicated siRNAs were immunostained with an antibody recognizing S6 phosphorylated at residues 235 and 236 (red channel) and stained with Hoechst to detect cell nuclei (blue channel). (C) GβL plays a role in cell size control. Shown are distributions of cell diameters of actively growing HEK-293T cells three days after transfection with siRNA targeting lamin (red line), mTOR or GβL (blue line). The mean ± S.D. (μm) of the cell diameters are: lamin siRNA 16.02 ± 0.05 (n=4); mTOR siRNA 15.47 ± 0.05 (*) (n=4); GβL siRNA 15.45 ± 0.06 (*) (n=4). (*) $p<0.05$ when compared to lamin control. (D) GβL stimulates the in vitro mTOR kinase activity. mTOR kinase activities towards GST-S6K1, 4E-BP1 and itself were determined using anti-myc immunoprecipitates prepared from cells transfected with the indicated plasmids. (E) GβL-mediated stimulation of the mTOR kinase activity correlates with the amount of GβL bound to mTOR and is independent of raptor. Myc-immonoprecipitates were prepared from HEK-293T cells expressing myc-mTOR and increasing amounts of HA-GβL and lysed in the Triton X-100-containing Buffer A (Kim et al., 2002). mTOR autophosphorylation and activity towards GST-S6K1 were determined as in (D). In (D) and (E), the numbers below the autoradiographs indicate the fold changes in ^{32}P incorporation as determined with a phospho-imager.

Figures 3A-3F show that GβL forms a heterotrimeric complex with raptor and the mTOR kinase domain. (A) The GβL-mTOR interaction is unaffected by nutrient conditions, rapamycin, and mitochondrial function. HEK-293T cells were not treated (control), deprived of leucine for 50 min (-leu), or deprived of leucine for 50 min and restimulated for 10 min with 52 μg/ml leucine (-leu +leu), or treated for 10 min with 0.001% ethanol, 20 nM rapamycin or 5 μM antimycin A. mTOR immunoprecipitates were prepared and analyzed by immunoblotting for mTOR, raptor, and GβL. (B)

Destabilization of the raptor-mTOR interaction by a reduction in GβL expression. mTOR immunoprecipitates and cell lysates prepared from cells transfected with siRNA targeting lamin or GβL were analyzed by immunoblotting for mTOR, raptor, and GβL. (C) GβL stabilizes the mTOR-raptor association. Immunoblotting was used to analyze the amounts of endogenous raptor and HA- GβL recovered in myc immunoprecipitates prepared from HEK-293T cells expressing myc-mTOR or myc-γ-tubulin with or without HA-GβL. (D) GβL has independent binding sites for mTOR and raptor. Immunoblotting was used to determine the amounts of endogenous mTOR and raptor in anti-myc immunoprecipitates prepared from HEK-293T cells expressing HA-tagged γ-tubulin, or wild-type or mutant GβL. (E) GβL promotes the binding of raptor to the mTOR kinase domain. Immunoblotting was used to determine the amounts of endogenous raptor and HA-tagged wild-type or mutant GβL in myc-immunoprecipitates prepaed from HEK-293T cells co-expressing a myc-mTOR fragment (amino acids 2115 to 2549) and the indicated GβL variants. (F) The capacity of GβL to stimulate the mTOR was co-expressed with HA-tagged wild-type or mutant GβL and its kinase activity determined as in Fig. 2D using myc-immunoprecipitates prepared from cells lysed in the Triton X-100-containing Buffer A (Kim et al., 2002).

Figures 4A-4E show that regulation of mTOR activity by raptor requires GβL. (A) Disruption of GβL-raptor interaction leads to an increase in S6K1 phosphorylation. Immunoblotting was used to determine the phosphorylation level of S6K1 in myc-immunoprecipitates prepared from HEK-293T cells co-expressing myc-S6K1 and the indicated HA-tagged variants of GβL. (B) The GβL-raptor interaction is inhibitory for S6K1 phosphorylation. The phosphorylation level of S6K1 was analyzed, as in (A), in myc-immunoprecipitates from HEK-293T cells co-expressing myc-S6K1 with HA-tagged wild type or mutant raptor (Kim et al., 2002), and wild type or mutant GβL. (C) The GβL-raptor interaction is part of the nutrient-sensitive mechanism necessary to stabilize the mTOR-raptor association. Cells co-expressing HA-mTOR with the indicated myc-tagged GβL variants were treated with (+) or without (-) DSP before lysis as described (Kim et al., 2002). Immunoblot analyses were used to determine the amounts of endogenous raptor and myc- GβL in HA-immunoprecipitates and the expression levels of myc-GβL in cellular lysates. (D) GβL and raptor have opposite

effects on the mTOR kinases activity. Myc-mTOR kinase activity toward GST-S6K1 was determined as in Fig. 2D from HEK-293T cells co-expressing myc-mTOR with the indicated HA-tagged GβL and/or raptor variants. (E) Model for regulation of mTOR by GβL and raptor.

Figures 5A-5C show the human p200 mRNA sequence (SEQ ID NO: 1).

Figures 6A-6C show the human p200 ORF sequence (SEQ ID NO: 2).

Figures 7A and 7B show the p200 amino acid sequence (SEQ ID NO: 3).

Figure 8 shows the human GβL mRNA sequence (SEQ ID NO: 4).

Figures 9A and 9B show the GβL ORF sequence without UTRs (SEQ ID NO: 5).

Figure 10 shows the GβL amino acid sequence (SEQ ID NO: 6).

Figures 11A-11D show that rictor is a novel mTOR-associated protein. (A) Silver stain of SDS-PAGE analysis of mTOR immunoprecipitates prepared from HeLa cells lysed in a CHAPS- or Triton X-100-containing buffer. (+) indicates inclusion of the blocking peptide for the mTOR antibody during the immunoprecipitation. The ~200 kDa band corresponds to rictor and a non-specific band (NS) obscures raptor. (B) Rictor homologues share common domain architectures. Analyses of indicated rictor homologues identified seven domains with sequence conservation and similar relative locations within each protein and are shown schematically as boxes. Domain five is repeated four times within each of the homologues and the multiple sequence alignment shows the sequence pattern of this repeat. Sequences with the following accession numbers were used to create the alignment: D. melanogaster, AAQ22398.1; A. gambiae, XP_309233.1; H. sapiens, AY515854; D. discoidieum, AAC35553.1; S. pombe, NP_596021.1; S. cerevisiae; NP_011018.1. (C) Specific interaction between endogenous mTOR and rictor. Immunoprecipitates prepared with the indicated antibodies were analyzed by immunoblotting for mTOR, rictor and raptor. Prior to use cells were treated with 5 μM Antimycin A for 15 min (Antimy), 20 nM rapamycin for 15 min (Rapa), deprived of leucine for 90 min (-Leu), or deprived of leucine and stimulated with 52 μg/ml leucine for 10 min (-Leu+Leu). (D) Endogenous mTOR interacts with recombinant rictor and raptor. Cellular lysates and mTOR immunoprecipitates prepared from HEK293T cells expressing myc-rictor, myc-raptor, or myc-GCP3 were analyzed by

immunoblotting for myc-tagged proteins. In parallel, anti-myc immunoprecipitates were analyzed by immunoblotting for mTOR.

Figures 12A-12C show that rictor and raptor define two distinct mTOR-containing complexes. (A) Immunoblot analyses for indicated proteins of mTOR immunoprecipitates and cell lysates prepared from HeLa, HEK293T, and DU145 cells. Equal amounts of total protein were analyzed from each cell type. (B) Immunoblot analyses for the presence of the indicated components of the mTOR signaling complex in immunoprecipitates prepared from HEK293T cell lysates with antibodies against rictor, mTOR, or raptor. (C) Recombinant wild type raptor but not a mutant raptor suppresses the binding of rictor to mTOR. mTOR immunoprecipitates prepared from HEK293T cells expressing the indicated tagged proteins were analyzed by immunoblotting with anti-myc and anti-HA antibodies.

Figures 13A-13E show that rictor does not participate in rapamycin-sensitive mTOR functions. (A) The sensitivity of the raptor-mTOR interaction to rapamycin depends on the presence of phosphate-containing molecules in the lysis buffer. mTOR immunoprecipitates prepared from cells treated with or without 20 nM rapamycin for 10 min and lysed in a phosphate-containing or phosphate-free buffer were analyzed by immunoblotting for the indicated proteins. (B) Raptor and mTOR, but not rictor, co-purify with FKBP12-rapamycin. Anti-HA immunoprecipitates prepared from HEK293T cells expressing HA-FKBP12 and treated with or without 20 nM rapamycin for 15 min were analyzed by immunoblotting for the indicated proteins. (C) Suppression of rictor expression slightly increases the amount of raptor in the mTOR complex and S6K1 activity. mTOR immunoprecipitates and cell lysates prepared from HEK293T or HeLa cells transfected with siRNAs targeting lamin or rictor were analyzed by immunoblotting for the indicated proteins. (D) Suppression of Drosophila rictor expression increases the phosphorylation state of dS6K. The indicated dsRNAs were applied to Drosophila S2 cells and cell lysates were analyzed by immunoblotting with the mammalian phospho-specific S6K1 and Drosophila S6K antibodies. (E) The rictor-containing mTOR complex does not phosphorylate S6K1. Immunoprecipitates prepared with the indicated antibodies were used in mTOR kinase assays using S6K1 as a substrate. Where indicated immunoprecipitates were treated with 100 nM FKBP12-rapamycin for 40 min before the

start of the assays. Immunoblotting was used to monitor the levels of rictor, mTOR, and raptor in the kinase reactions.

Figures 14A-14C show that rictor and mTOR, but not raptor, regulate the PKCα phosphorylation state in human and Drosophila cells. (A) siRNA-mediated reduction in the expression of total PKCα in HeLa cells also reduces the immunoblot signal from a phosphospecific antibody recognizing phospho-S657 of PKCα but does not affect the levels of S6K1. (B) Immunoblotting was used to analyze the phosphorylation states of PKCα and S6K1 in HeLa cells with reduced expression of rictor, raptor, or mTOR or treated with rapamycin. Lentiviruses were used to express siRNAs targeting rictor, raptor, mTOR or luciferase. (C) dsRNAs corresponding to the genes for the indicated proteins were applied to S2 Drosophila cells. After 4 days lysates were prepared and analyzed by immunoblotting for dPKCα and phospho-dPKCα levels.

Figures 15A-15C show that rictor, mTOR and PKCα regulate the organization of the actin cytoskeleton. (A) Staining for actin (red), paxillin (green) and DNA (blue) reveals the organization of the actin cytoskeleton in HeLa cells transduced with the siRNA-expressing lentiviruses described in Figure 14A. Arrows point to bundles of actin fibers. Images captured with a 60x objective are shown. (B) Higher magnification of portions of the merged images from Figure 15A. (C) Like cells with reduced rictor expression, cells with reduced expression of PKCα have an altered actin cytoskeleton.

Figures 16A-16D that mTOR regulates the rictor phosphorylation state. (A) HeLa cells with reduced expression of mTOR or of a control protein were metabolically labeled with ^{32}P and the level of phosphorylated rictor determined by immunoprecipitation followed by autoradiography and immunoblotting for the indicated proteins. (B) The mobility of rictor in SDS-PAGE is affected by mTOR. HeLa cells with siRNA-mediated reductions in mTOR or controls were analyzed by immunoblotting for mTOR, rictor, and raptor. (C) The phosphorylation state of rictor affects its mobility in SDS-PAGE. Rictor immunoprecipitates were incubated with or without calf intestinal phosphatase (CIP) or heat inactivated CIP and analyzed by SDS-PAGE and immunoblotting for rictor. (D) Osmotic stress increases the mobility of rictor in SDS-PAGE. Lysates of HeLa cells exposed for 1 hr to 20 nM rapamycin, 20 nM LY294002, 100 mM 2 deoxyglucose (2-DG), medium without leucine or glucose, or medium without leucine or glucose followed

by the readdition of the missing component for 10 minutes were analyzed by immunoblotting for rictor.

Figure 17 shows the amino acid sequence of the GβL protein and the seven WD40 repeats in this protein.

Figure 18 shows the structural model of the GβL protein.

Figure 19 shows that GβL interacts with the mTOR kinase domain. Myc-tagged full-length mTOR, its indicated fragments, or γ-tubulin were coexpressed with HA-GβL, and anti-myc immunoprecipitates were analyzed by anti-HA and anti-myc immunoblotting.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based in part on Applicants' discovery that two proteins (designated GβL and rictor) associate with the mTOR kinase and participate in the mTOR signaling pathway. As described herein, the term "mTOR-associated protein" or "mTOR-AP" refers to a protein capable of interacting with and/or binding to an mTOR polypeptide. Generally, the mTOR-AP may interact directly or indirectly with the mTOR polypeptide. According to the application, specific types of the mTOR-AP are GβL polypeptides and rictor polypeptides.

In one aspect, the present invention relates to a GβL protein. As described herein, GβL acts to positively regulate the mTOR pathway by stimulating (activating) mTOR kinase activity and also mediates raptor-mTOR interaction. This protein interacts strongly with the C-terminal portion of mTOR (approximately amino acid residues 1348-2549) but does not interact with the N-terminal amino acid residues of mTOR. GβL has been shown to form a complex with mTOR (a GβL-mTOR complex) and, complex with mTOR and raptor (a GβL-mTOR-raptor complex). In the GβL-mTOR complex, GβL is linked to the mTOR kinase domain. Through its role as an activator of mTOR kinase activity, GβL plays an essential positive role in controlling cell growth and, thus, is a target that can, in turn, be regulated in order to alter (enhance or decrease) cell growth.

Certain embodiments of the present invention provide isolated GβL, alone, in complex with mTOR (linked to the mTOR kinase domain to form a GβL-mTOR complex) or in complex with mTOR kinase and raptor (a GβL-mTOR-raptor complex),

the formation of which is necessary for raptor to inhibit the mTOR pathway (inhibit mTOR kinase activity). As shown herein, GβL mediates interaction between raptor and the mTOR kinase domain.

In another aspect, the present invention relates to a rictor protein (also referred to as a p200 protein). As described herein, mTOR also exists as part of a distinct complex defined by the novel protein rictor (rapamycin insensitive companion of mTOR). For example, the rictor-containing mTOR complex contains GβL but not raptor and it neither regulates the mTOR effector S6K1 nor is it a target of rapamycin. Through a rapamycin-insensitive and raptor-independent pathway, the rictor-containing complex modulates the phosphorylation state of Protein Kinase C alpha (PKCα) and the organization of the actin cytoskeleton. Thus, the mTOR pathway has both rapamycin-sensitive and rapamycin-insensitive functions. The latter one may be unrelated to mass accumulation and of different therapeutic interest.

Certain embodiments of the present invention provide isolated rictor, alone, in complex with mTOR, or in complex with mTOR kinase and GβL, the formation of which is necessary for rictor to modulate signaling through the mTOR pathway, such as phosphorylation of PKCα and the organization of the actin cytoskeleton.

Raptor (*r*egulated *a*ssociated *p*rotein of *TOR*) is a subunit of an mTOR-containing complex whose association with mTOR is modulated by nutrients and which regulates the mTOR kinase activity (Kim, D. H., et al. (2002), Cell *110*, 163-75). Hara et al. independently identified the same interacting protein and adopted the raptor name (Hara, K., et al. (2002), Cell *110*, 177-89). Raptor is a large protein of 149 kDa in molecular weight and contains an N-terminal RNC (Raptor N-terminal Conserved) domain found in all its eukaryotic homologues, three HEAT repeats following the RNC, and seven WD-40 repeats in the C-terminal third of the protein (Kim, D. H., et al. (2002), Cell *110*, 163-75). The mTOR-binding site on raptor is not easily defined and, based on mutagenesis and truncation studies, may require the overall confrontation of raptor and/or multiple contacts between the proteins. Previously, Applicants proposed that raptor has at least two functions in the mTOR pathway. It clearly has a positive role within cells in maintaining an active mTOR pathway, as revealed by the inhibitory effects of reducing raptor expression on S6K1 activity and cell size (Kim, D. H., et al. (2002), Cell *110*, 163-

75). Raptor over-expression also stimulates the kinase activity of recombinant mTOR towards S6K1 and 4E-BP1, suggesting that raptor may be an adaptor protein that recruits mTOR substrates to the mTOR kinase domain (Hara, K., et al. (2002), Cell *110*, 177-89). In addition to a positive function for a raptor, Applicants proposed that raptor also negatively regulates the mTOR kinase. Conditions that inhibit the pathway, such as nutrient deprivation, stabilized the raptor-mTOR association and inhibited the kinase activity of endogenous mTOR, and raptor over expression decreased the phosphorylation state of S6K1 within cells and the mTOR kinase activity in vitro (Kim, D. H., et al. (2002), Cell *110*, 163-75). These results imply that regulation of mTOR kinase activity is complex and may be difficult to understand without knowledge of how mTOR and raptor interact with each other or with unidentified components of the complex that may affect the regulatory function(s) of raptor.

Exemplary mTOR-AP nucleic acids and polypeptides

The present invention provides nucleic acids and the polypeptides encoded thereby relating to two mTOR-associated proteins (mTOR-APs), termed GβL and rictor. Described herein are isolated GβL polypeptides and rictor polypeptides, fragments and variants thereof; isolated nucleic acids (e.g., DNA, RNA) encoding GβL and rictor polypeptides, fragments and variants thereof; methods of producing GβL and rictor polypeptides; and methods in which GβL and rictor polypeptides are used. Such nucleic acids and polypeptides are of eukaryotic origin, such as mammalian origin (e.g., mouse or human).

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides. The terms "protein" and "polypeptide" are used interchangeably herein.

In some aspects, the invention provides GβL and rictor nucleic acid sequences and proteins encoded thereby, as well as oligonucleotides that are portions of the nucleic acid sequences, antibodies that bind the encoded proteins, screening assays to identify

agents that modulate activity of GβL, rictor or both, and/or biological events affected by GβL, rictor or both. These compounds may be used in the treatment and/or prophylaxis of diseases that are responsive to mTOR-AP modulation, for example, cancer and diabetes.

In one aspect, the invention provides an isolated nucleic acid comprising a nucleic acid which hybridizes under high stringency conditions to a nucleic acid having the sequence of SEQ ID NO: 2 or a sequence complementary thereto or having the sequence of SEQ ID NO: 4 or a sequence complementary thereto. In a further embodiment, the invention is an isolated nucleic acid that is at least about 70%, 80%, 90%, 95%, 97-98%, or greater than 99% identical to a sequence corresponding to at least about 12, at least about 15, at least about 25, at least about 40, at least about 100, at least about 300, at least about 500, at least about 1000, or at least about 2100 consecutive nucleotides up to the full length of SEQ ID NO: 2 or 4, or a sequence complementary thereto.

In one specific embodiment, nucleic acids exhibit one of the foregoing levels of identity to SEQ ID NO: 1 or 2 and encode polypeptides that also exhibit substantially the same activity or function as a rictor protein encoded by SEQ ID NO: 3. In another specific embodiment, nucleic acids exhibit one of the foregoing levels of identity to SEQ ID NO: 4 or 5 and encode polypeptides that also exhibit substantially the same activity or function as a GβL protein encoded by SEQ ID NO: 6.

Isolated nucleic acids of the present invention are substantially free from unrelated nucleic acids as well as contaminating polypeptides, nucleic acids and other cellular material that normally are associated with the nucleic acid in a cell or that are associated with the nucleic acid in a library.

In other embodiments, the invention provides expression vectors (constructs) comprising: (a) a nucleic acid which hybridizes under high stringency conditions to a sequence of SEQ ID NO: 2 or 4, or a nucleotide sequence that is at least about 70%, 80%, 90%, 95%, 97-98%, or greater than 99% identical to a sequence that is at least about 12, at least about 15, at least about 25, at least about 40, at least about 100, at least about 300, at least about 500, at least about 1000, or at least about 2100 consecutive nucleotides up to the full length of SEQ ID NO: 2 or 4, or a sequence complementary thereto; and (b) a transcriptional regulatory sequence operably linked to the nucleotide sequence. In certain

embodiments, an expression vector of the present invention additionally comprises a transcriptional regulatory sequence, e.g., at least one of a transcriptional promoter or transcriptional enhancer sequence, which regulatory sequence is operably linked to a rictor or GβL sequence. In another embodiment, the nucleic acid may be included in an expression vector capable of replicating in and expressing the encoded rictor or GβL polypeptide in a prokaryotic or eukaryotic cell. In a related embodiment, the invention provides a host cell transfected with the expression vector.

Any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding a rictor or GβL polypeptide. Such useful expression control sequences include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

As will be apparent, the subject gene constructs can be used to express rictor or GβL polypeptides in cells propagated in culture, e.g., to produce proteins or polypeptides, including fusion proteins or polypeptides, for purification.

This invention also pertains to a host cell transfected with a recombinant gene comprising a coding sequence for the subject rictor or GβL polypeptides. The host cell may be any prokaryotic or eukaryotic cell. For example, a polypeptide of the present invention may be expressed in bacterial cells (e.g., E. coli), insect cells (e.g., using a

baculovirus expression system), yeast, avian, or mammalian cells (e.g., human cells such as 293T, HeLa).

Accordingly, the present invention further pertains to methods of producing the subject rictor or GβL polypeptides. For example, a host cell transfected with an expression vector encoding a rictor or GβL polypeptide can be cultured under appropriate conditions to allow expression of the polypeptide to occur. The polypeptide may be secreted and isolated from a mixture of cells and medium containing the polypeptide. Alternatively, the polypeptide may be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for particular epitopes of the polypeptide. In one embodiment, the rictor or GβL polypeptide is a fusion protein containing a domain which facilitates its purification, such as a rictor-GST or GβL-GST fusion protein, rictor-intein or GβL-intein fusion protein, rictor-cellulose binding domain or GβL-cellulose binding domain fusion protein, and rictor-polyhistidine or GβL-polyhistidine fusion protein.

A nucleotide sequence encoding a rictor or GβL polypeptide can be used to produce a recombinant form of the protein via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial) cells, are standard procedures.

A recombinant rictor or GβL nucleic acid can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vehicles for production of recombinant rictor or GβL polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a rictor or GβL polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as E. coli.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into S. cerevisiae. These vectors can replicate in E. coli due to the presence of the pBR322 ori, and in S. cerevisiae due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used.

Certain mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. In some instances, it may be desirable to express the recombinant rictor or GβL polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. This type of expression system can be useful under conditions where it is desirable, e.g., to produce an immunogenic fragment of a rictor or GβL polypeptide. For example, the VP6 capsid protein of rotavirus can be used as an immunologic carrier protein for portions of polypeptide, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the portion of the rictor or GβL polypeptide to which antibodies are to be raised can be incorporated into a fusion gene construct

which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising a portion of the protein as part of the virion. The Hepatitis B surface antigen can also be utilized in this role as well. Similarly, chimeric constructs coding for fusion proteins containing a portion of a rictor or GβL polypeptide and the poliovirus capsid protein can be created to enhance immunogenicity.

In yet another embodiment, the invention provides a substantially pure nucleic acid which hybridizes under high stringency conditions to a nucleic acid probe that comprises at least about 12, at least about 15, at least about 25, or at least about 40 consecutive nucleotides up to the full length of SEQ ID NO: 2 or 4, or a sequence complementary thereto or up to the full length of the gene of which said sequence is a fragment. The invention also provides an antisense oligonucleotide analog which hybridizes under stringent conditions to at least 12, at least 25, or at least 50 consecutive nucleotides up to the full length of SEQ ID NO: 2 or 4, or a sequence complementary thereto.

One of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature. In another embodiment, the invention provides nucleic acids which hybridize under high stringency conditions of 0.5 x SSC at 60 °C followed by 2 washes at 0.5 x SSC at 60 °C.

In a further embodiment, the invention provides a nucleic acid comprising a nucleic acid encoding the amino acid sequence of SEQ ID NO: 3 or 6, or a nucleic acid

complementary thereto. In a further embodiment, the encoded amino acid sequence is at least about 70%, 80%, 90%, 95%, or 97-98%, or greater than 99% identical to a sequence corresponding to at least about 12, at least about 15, at least about 25, or at least about 40, at least about 100, at least about 200, at least about 300, at least about 400 or at least about 500 consecutive amino acid residues up to the full length of SEQ ID NO: 3 or 6.

Nucleic acids of the invention further include nucleic acids that comprise variants of SEQ ID NO: 2 or 4. Variant nucleotide sequences include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants; and will, therefore, include coding sequences that differ from the nucleotide sequence of the coding sequence designated in SEQ ID NO: 2 or 4, e.g., due to the degeneracy of the genetic code. In other embodiments, variants will also include sequences that will hybridize under highly stringent conditions to a nucleotide sequence of a coding sequence designated in SEQ ID NO: 2 or 4.

Isolated nucleic acids which differ from SEQ ID NO: 2 or 4 due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. All such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

In another embodiment, the invention provides a probe or primer (e.g., DNA, RNA) which hybridizes under stringent conditions to at least about 12, at least about 15, at least about 25, or at least about 40 consecutive nucleotides of sense or antisense sequence selected from SEQ ID NO: 2 or 4, or a sequence complementary thereto. In certain embodiments, a probe of the present invention hybridizes to a characteristic region of SEQ ID NO: 2 or 4 and is useful to identify additional toll-like receptors. The probe may include a detachable label, such as a radioisotope, a fluorescent compound, an

enzyme, or an enzyme co-factor. The invention further provides arrays of at least about 10, at least about 25, at least about 50, or at least about 100 different probes as described above attached to a solid support. Such arrays are useful to assess samples (e.g., tissues, blood, cells) for the presence of rictor or GβL nucleic acids (e.g., rictor mRNA or GβL mRNA).

Optionally, a rictor or GβL nucleic acid of the invention will genetically complement a partial or complete rictor or GβL loss of function phenotype in a cell. For example, a rictor or GβL nucleic acid of the invention may be expressed in a cell in which endogenous rictor or GβL gene expression has been reduced by RNAi, and the introduced rictor or GβL nucleic acid will mitigate a phenotype resulting from the RNAi. The term "RNA interference" or "RNAi" refers to any method by which expression of a gene or gene product is decreased by introducing into a target cell one or more double-stranded RNAs which are homologous to the gene of interest (particularly to the messenger RNA of the gene of interest).

Another aspect of the invention relates to rictor or GβL nucleic acids that are used for antisense, RNAi or ribozymes. As used herein, nucleic acid therapy refers to administration or in situ generation of a nucleic acid or a derivative thereof which specifically hybridizes (e.g., binds) under cellular conditions with the cellular mRNA and/or genomic DNA encoding one of the subject rictor or GβL polypeptides so as to inhibit production of that protein, e.g., by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix.

A nucleic acid therapy construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a rictor or GβL polypeptide. Alternatively, the construct is an oligonucleotide which is generated ex vivo and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences encoding a rictor or GβL polypeptide. Such oligonucleotide probes are optionally modified oligonucleotides which are resistant to endogenous nucleases, e.g., exonucleases and/or endonucleases,

and are therefore stable in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in nucleic acid therapy have been reviewed, for example, by van der Krol et al., (1988) Biotechniques 6:958-976; and Stein et al., (1988) Cancer Res 48:2659-2668. Nucleic acid constructs of the invention are useful in therapeutic, diagnostic, and research contexts.

In addition to their use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the rictor or GβL DNA or RNA sequences to which they specifically bind, such as for determining the level of expression of a gene of the invention or for determining whether a gene of the invention contains a genetic lesion.

In another aspect, the invention provides polypeptides. In one embodiment, the invention pertains to a polypeptide encoded by a nucleic acid which hybridizes under stringent conditions to a nucleic acid of SEQ ID NO: 2 or 4, a sequence complementary thereto, or a fragment encoding an amino acid sequence comprising at least about 25, or at least about 40 amino acid residues thereof.

In another embodiment, the rictor or GβL polypeptide comprises a sequence that is identical with or homologous to SEQ ID NO: 3 or 6. For instance, a rictor or GβL polypeptide preferably has an amino acid sequence at least 70% identical to a polypeptide represented by SEQ ID NO: 3 or 6 or an amino acid sequence that is 80%, 90% or 95% identical thereto. The rictor or GβL polypeptide can be full-length, such as the polypeptide represented by the amino acid sequence in SEQ ID NO: 3 or 6, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100, 150, 200, 250, 300, 400 or 500 or more amino acid residues in length.

In another embodiment, the invention features a purified or recombinant polypeptide fragment of a rictor or GβL polypeptide, which polypeptide has the ability to modulate (e.g., stimulate or antagonize) an activity of a wild-type rictor or GβL protein. In one embodiment, the polypeptide fragment comprises a sequence identical or homologous to the amino acid sequence designated in SEQ ID NO: 3 or 6.

Moreover, as described below, the rictor or GβL polypeptide can be either an agonist or alternatively, an antagonist of a biological activity of a naturally occurring form of the protein, e.g., the polypeptide is able to modulate the intrinsic biological activity of a rictor or GβL protein. Optionally, the subject rictor or GβL polypeptide is able to modulate signaling through a complex containing an mTOR protein and a rictor protein, or through a complex containing an mTOR protein and a GβL protein. Signaling through such complexes include, but are not limited to, activation of mTOR kinase activity, phosporylation of S6 kinase 1 (S6K1), phosphorylation of protein kinase C α (PKC α), and organization of the actin cytoskeleton.

The present invention also relates to chimeric molecules, such as fusion proteins, that comprise all or a portion of a rictor or GβL polypeptide and a second polypeptide that is heterologous (not a rictor or GβL polypeptide), such as the extracellular domain of a CD4 receptor or an epitope tag, such as a Flag or myc epitope tag.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature.

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al., John Wiley & Sons: 1992).

The present invention also makes available isolated and/or purified forms of the subject rictor or GβL polypeptides, which are isolated from, or otherwise substantially

free of, other intracellular proteins which might normally be associated with the protein or a particular complex including the protein. Rictor or GβL polypeptides which are recombinantly produced (e.g., by recombinant DNA methods) or chemically synthesized are also the subject of this invention.

Optionally, a rictor or GβL polypeptide of the invention will function in place of an endogenous rictor or GβL polypeptide, respectively, for example by mitigating a partial or complete rictor or GβL loss of function phenotype in a cell.

Variants and fragments of a rictor or GβL polypeptide may have enhanced activity or constitutive activity, or, alternatively, act to prevent rictor or GβL polypeptides from performing one or more functions. For example, a truncated form lacking one or more domains may have a dominant negative effect.

Another aspect of the invention relates to polypeptides derived from a full-length rictor or GβL polypeptide. Isolated peptidyl portions of the subject proteins can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such polypeptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, the subject protein can be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of the formation of a specific protein complex, or more generally of a rictor- or GβL-containing complex, such as by microinjection assays.

It is also possible to modify the structure of the subject rictor or GβL polypeptides for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo). Such modified polypeptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the rictor or GβL polypeptides described in more detail herein. Such modified polypeptides include peptide mimetics. Peptide mimetics include chemically modified peptides and peptide-like molecules

containing non-naturally occurring amino acids. Modified polypeptides can also be produced, for instance, by amino acid substitution, deletion, or addition.

For instance, it is reasonable to expect, for example, that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (e.g., conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Whether a change in the amino acid sequence of a polypeptide results in a functional homolog can be readily determined by assessing the ability of the variant polypeptide to produce a response in cells in a fashion similar to the wild-type protein. For instance, such variant forms of a rictor or GβL polypeptide can be assessed, e.g., for their ability to activate the mTOR kinase; e.g., to stimulate phosphorylation of S6K1; to stimulate phosphorylation of PKCα; to modulate organization of the actin cytoskeleton; or to bind to another polypeptide such as for example, an mTOR polypeptide. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method of generating sets of combinatorial mutants of the subject mTOR-AP (e.g., rictor or GβL) polypeptides, as well as truncation mutants, and is especially useful for identifying potential variant sequences (e.g., homologs) that are functional in binding to a rictor or GβL polypeptide. The purpose of screening such combinatorial libraries is to generate, for example, rictor or GβL homologs which can act as either agonists or antagonists, or alternatively, which possess novel activities all together. Combinatorially-derived homologs can be generated which have a selective potency relative to a naturally occurring rictor or GβL polypeptide. Such proteins, when expressed from recombinant DNA constructs, can be used in gene therapy protocols.

Yet another aspect of the present invention concerns an immunogen which comprises an mTOR-AP (e.g., rictor or GβL) polypeptide capable of eliciting an immune response specific for the mTOR-AP polypeptide; e.g., a humoral response, an antibody response; or a cellular response. In certain embodiments, the immunogen comprises an

antigenic determinant, e.g., a unique determinant, from a protein represented by SEQ ID NO: 3 or 6.

Antibodies

Another aspect of the invention pertains to an antibody specifically reactive with an mTOR-AP (e.g., rictor or GβL) polypeptide. For example, by using immunogens derived from a rictor or GβL polypeptide, e.g., based on the cDNA sequences, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols. A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide (e.g., an mTOR-AP polypeptide or an antigenic fragment which is capable of eliciting an antibody response, or a fusion protein as described above). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of a rictor or GβL polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies. In one embodiment, the subject antibodies are immunospecific for antigenic determinants of a rictor or GβL polypeptide of a mammal, e.g., antigenic determinants of a protein set forth in SEQ ID NO: 3 or 6.

In another embodiment, the antibodies are immunoreactive with one or more proteins having an amino acid sequence that is at least 70% identical, at least 80% identical to an amino acid sequence as set forth in SEQ ID NO: 3 or 6. In other embodiments, an antibody is immunoreactive with one or more proteins having an amino acid sequence that is 75%, 80%, 85%, 90%, 95%, 98%, 99% or identical to an amino acid sequence as set forth in SEQ ID NO: 3 or 6.

Following immunization of an animal with an antigenic preparation of an mTOR-AP (e.g., rictor or GβL) polypeptide, anti-rictor or anti-GβL antisera can be obtained and, if desired, polyclonal anti-rictor or anti-GβL antibodies isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are

well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) Nature, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) Immunology Today, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with a mammalian rictor or GβL polypeptide of the present invention and monoclonal antibodies isolated from a culture comprising such hybridoma cells. In one embodiment, anti-mouse rictor or anti-mouse GβL antibodies specifically react with the protein encoded by a nucleic acid having SEQ ID NO: 2 or 4, respectively.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with one of the subject mTOR-AP (e.g., rictor or GβL) polypeptides. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab)_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab)_2$ fragment can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention is further intended to include bispecific, single-chain, and chimeric and humanized molecules having affinity for a rictor or GβL polypeptide conferred by at least one CDR region of the antibody. In certain embodiments, the antibody further comprises a label attached thereto and able to be detected, (e.g., the label can be a radioisotope, fluorescent compound, enzyme or enzyme co-factor).

An application of anti-rictor or anti-GβL antibodies of the present invention is in the immunological screening of cDNA libraries constructed in expression vectors such as gt11, gt18-23, ZAP, and ORF8. Messenger libraries of this type, having coding sequences inserted in the correct reading frame and orientation, can produce fusion proteins. For instance, gt11 will produce fusion proteins whose amino termini consist of ß-galactosidase amino acid sequences and whose carboxy termini consist of a foreign polypeptide. Antigenic epitopes of a rictor or GβL polypeptide, e.g., other orthologs of a particular protein or other paralogs from the same species, can then be detected with antibodies, as, for example, reacting nitrocellulose filters lifted from infected plates with

the appropriate anti-rictor or anti-GβL antibodies. Positive phage detected by this assay can then be isolated from the infected plate. Thus, the presence of rictor or GβL homologs can be detected and cloned from other animals, including humans.

Transgenic Animals

Another aspect of the invention features transgenic non-human animals which express a heterologous mTOR-AP (rictor or GβL) gene, e.g., having a sequence of SEQ ID NO: 2 or 5, or fragments thereof. In another aspect, the invention features transgenic non-human animals which have had one or both copies of the endogenous mTOR-AP gene disrupted in at least one of the tissue or cell-types of the animal. In one embodiment, the transgenic non-human animals is a mammal such as a mouse, rat, rabbit, goat, sheep, dog, cat, cow or non-human primate. Without being bound to theory, it is proposed that such an animal may display a phenomenon associated with reduced or increased chance of a disease or a condition, such as cancer, diabetes, or cardiovascular diseases. Accordingly, such a transgenic animal may serve as a useful animal model to study the progression of such diseases or conditions.

The term "transgene" is used herein to describe genetic material that has been or is about to be artificially inserted into the genome of a mammalian cell, particularly a mammalian cell of a living animal. The transgene is used to transform a cell, meaning that a permanent or transient genetic change, preferably a permanent genetic change, is induced in a cell following incorporation of exogenous DNA. A permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACs, and the like. Of interest are transgenic mammals. Preferably, the transgenic-animals are mice.

Transgenic animals comprise an exogenous nucleic acid sequence present as an extrachromosomal element or stably integrated in all or a portion of its cells, especially in germ cells. Unless otherwise indicated, it will be assumed that a transgenic animal comprises stable changes to the germline sequence. During the initial construction of the animal, "chimeras" or "chimeric animals" are generated, in which only a subset of cells have the altered genome. Chimeras are primarily used for breeding purposes in order to

generate the desired transgenic animal. Animals having a heterozygous alteration are generated by breeding of chimeras. Male and female heterozygotes are typically bred to generate homozygous animals.

The exogenous gene is usually either from a different species than the animal host, or is otherwise altered in its coding or non-coding sequence. The introduced gene may be a wild-type gene, naturally occurring polymorphism, or a genetically manipulated sequence, for example having deletions, substitutions or insertions in the coding or non-coding regions. Where the introduced gene is a coding sequence, it is usually operably linked to a promoter, which may be constitutive or inducible, and other regulatory sequences required for expression in the host animal.

In one aspect of the invention, an mTOR-AP (rictor or GβL) transgene can encode the wild-type form of the protein, homologs thereof, as well as antisense constructs. An mTOR-AP transgene can also encode a soluble form of an mTOR-AP, e.g., one that has tumor suppressor activity.

It may be desirable to express the heterologous mTOR-AP transgene conditionally such that either the timing or the level of mTOR-AP gene expression can be regulated. Such conditional expression can be provided using prokaryotic promoter sequences which require prokaryotic proteins to be simultaneous expressed in order to facilitate expression of the mTOR-AP transgene. Exemplary promoters and the corresponding trans-activating prokaryotic proteins are given in U.S. Patent No. 4,833,080.

Moreover, transgenic animals exhibiting tissue specific expression can be generated, for example, by inserting a tissue specific regulatory element, such as an enhancer, into the transgene. For example, the endogenous mTOR-AP gene promoter or a portion thereof can be replaced with another promoter and/or enhancer, e.g., a CMV or a Moloney murine leukemia virus (MLV) promoter and/or enhancer.

Transgenic animals containing an inducible mTOR-AP transgene can be generated using inducible regulatory elements (e.g., metallothionein promoter), which are well-known in the art. mTOR-AP transgene expression can then be initiated in these animals by administering to the animal a compound which induces gene expression (e.g.,

heavy metals). Another preferred inducible system comprises a tetracycline-inducible transcriptional activator (U.S. Patent Nos. 5,654,168 and 5,650,298).

The present invention provides transgenic animals that carry the transgene in all their cells, as well as animals that carry the transgene in some, but not all cells, i.e., mosaic animals. The transgene can be integrated as a single transgene or in tandem, e.g., head to head tandems, or head to tail or tail to tail or as multiple copies.

The successful expression of the transgene can be detected by any of several means well known to those skilled in the art. Non-limiting examples include Northern blot, in situ hybridization of mRNA analysis, Western blot analysis, immunohistochemistry, and FACS analysis of protein expression.

In a further aspect, the invention features non-human animal cells containing an mTOR-AP transgene, preferentially a human mTOR-AP transgene. For example, the animal cell (e.g., somatic cell or germ cell) can be obtained from the transgenic animal. Transgenic somatic cells or cell lines can be used, for example, in drug screening assays. Transgenic germ cells, on the other hand, can be used in generating transgenic progeny.

Although not necessary to the operability of the invention, the transgenic animals described herein may comprise alterations to endogenous genes in addition to, or alternatively, to the genetic alterations described above. For example, the host animals may be either "knockouts" or "knockins" for the mTOR-AP gene. Knockouts have a partial or complete loss of function in one or both alleles of an endogenous gene of interest. Knockins have an introduced transgene with altered genetic sequence and/or function from the endogenous gene. The two may be combined, for example, such that the naturally occurring gene is disabled, and an altered form introduced. For example, it may be desirable to knockout the host animal's endogenous mTOR-AP gene, while introducing an exogenous mTOR-AP gene (e.g., a human mTOR-AP gene).

In a knockout, preferably the target gene expression is undetectable or insignificant. For example, a knock-out of an mTOR-AP gene means that function of the mTOR-AP has been substantially decreased so that expression is not detectable or only present at insignificant levels. This may be achieved by a variety of mechanisms, including introduction of a disruption of the coding sequence, e.g., insertion of one or more stop codons, insertion of a DNA fragment, deletion of coding sequence, substitution

of stop codons for coding sequence, etc. In some cases, the exogenous transgene sequences are ultimately deleted from the genome, leaving a net change to the native sequence. Different approaches may be used to achieve the "knock-out." A chromosomal deletion of all or part of the native gene may be induced, including deletions of the non-coding regions, particularly the promoter region, 3' regulatory sequences, enhancers, or deletions of gene that activate expression of APP genes. A functional knock-out may also be achieved by the introduction of an anti-sense construct that blocks expression of the native genes (for example, see Li and Cohen (1996) Cell 85:319-329). "Knock-outs" also include conditional knock-outs, for example, where alteration of the target gene occurs upon exposure of the animal to a substance that promotes target gene alteration, introduction of an enzyme that promotes recombination at the target gene site (e.g., Cre in the Cre-lox system), or other method for directing the target gene alteration postnatally.

A "knockin" of a target gene means an alteration in a host cell genome that results in altered expression or function of a native target gene. Increased (including ectopic) or decreased expression may be achieved by introduction of an additional copy of the target gene, or by operatively inserting a regulatory sequence that provides for enhanced expression of an endogenous copy of the target gene. These changes may be constitutive or conditional, i.e., dependent on the presence of an activator or repressor. The use of knockin technology may be combined with production of exogenous sequences to produce the transgenic animals of the invention.

Drug Screening Assays

In certain embodiments, the present invention provides assays for identifying therapeutic agents which either interfere with or promote function of the mTOR-AP protein (e.g., rictor or GβL). In certain embodiments, agents of the invention specifically modulate rictor activity. In another embodiment, agents of the invention specifically modulate GβL activity. In certain embodiments, agents of the invention modulate the activity of rictor and/or GβL and may be used to treat certain diseases such as cancer or diabetes, or a disease or condition that is responsive to modulation of the mTOR or mTOR-AP (e.g., rictor or GβL). In certain embodiments, the invention provides assays

to identify, optimize or otherwise assess agents that increase or decrease the activity of a rictor polypeptide, a GβL polypeptide, or both a rictor and a GβL polypeptide.

In certain embodiments, an assay of the invention comprises screening for activation of mTOR kinase. For example, mammalian cells such as HeLa cells are contacted with a compound, and then lysed. mTOR kinases are then immunoprecipitated and assayed for its activation. See for example, Example 6 as described below.

In certain embodiments, an assay as described above may be used to identify agents that modulate the anti-tumor activity of an mTOR-AP (e.g., rictor or GβL). Alternative, the present invention contemplates a screening assay that generally involves adding a test agent to an assay designed to assess the anti-tumor activity of a rictor or GβL polypeptide. The parameters detected in a screening assay may be compared to a suitable reference. A suitable reference may be an assay run previously, in parallel or later that omits the test agent. A suitable reference may also be an average of previous measurements in the absence of the test agent. In general, the components of a screening assay mixture may be added in any order consistent with the overall activity to be assessed, but certain variations may be preferred.

In a screening assay, the effect of a test agent may be assessed by, for example, assessing the effect of the test agent on kinetics, steady-state and/or endpoint of the reaction.

Certain embodiments of the invention relate to assays for identifying agents that bind to an mTOR-AP polypeptide (e.g., rictor or GβL), optionally a particular domain of rictor or GβL, such as a domain that binds to an mTOR protein. In certain embodiments, the invention relates to assays for identifying agents that bind to both a rictor and a GβL polypeptide. A wide variety of assays may be used for this purpose, including labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, and immunoassays for protein binding. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for modeling intermolecular interactions and design of test agents. In one embodiment, an assay detects agents which inhibit the activation of one or more subject rictor and/or GβL polypeptides. In another embodiment, the assay detects agents which modulate the intrinsic biological activity of a rictor and/or GβL polypeptide, such as binding to an

mTOR protein, activation of the mTOR kinase, or stimulating phosphorylation of PKCα, or modulating the actin cytoskeleton.

In additional embodiments of the invention, assay formats include those which approximate such conditions as formation of protein complexes, enzymatic activity, and rictor or GβL antitumor activity, e.g., purified proteins or cell lysates, as well as cell-based assays which utilize intact cells. Simple binding assays can also be used to detect agents which bind to rictor and/or GβL. Such binding assays may also identify agents that act by disrupting the interaction of a rictor or a GβL polypeptide with an mTOR protein. Agents to be tested can be produced, for example, by bacteria, yeast or other organisms (e.g., natural products), produced chemically (e.g., small molecules, including peptidomimetics), or produced recombinantly. In one embodiment, the test agent is a small organic molecule having a molecular weight of less than about 2,000 daltons.

In a further embodiment, the invention provides an assay for identifying a test compound which inhibits or potentiates the activation of a rictor and/or GβL polypeptide, comprising: (a) forming a reaction mixture including a rictor or GβL polypeptide and a test compound; and (b) detecting activation of said rictor or GβL polypeptides; wherein a change in the activation of said rictor or GβL polypeptide in the presence of the test compound, relative to activation in the absence of the test compound, indicates that said test compound potentiates or inhibits activation of said rictor or GβL polypeptide.

Assaying rictor-containing or GβL-containing complexes (e.g., a complex comprising an mTOR protein and a rictor protein, or a complex comprising an mTOR protein and a GβL protein) in the presence and absence of a candidate inhibitor, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes.

In one embodiment of the present invention, drug screening assays can be generated which detect inhibitory agents on the basis of their ability to interfere with assembly or stability of a rictor-containing or GβL-containing complex. In an exemplary binding assay, the compound of interest is contacted with a mixture comprising a rictor or GβL polypeptide and at least one interacting polypeptide such as an mTOR protein. Detection and quantification of rictor-containing or GβL-containing complexes provides a means for determining the compound's efficacy at inhibiting (or potentiating)

interaction between the two polypeptides. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, the formation of complexes is quantitated in the absence of the test compound.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays of the present invention which are performed in cell-free systems, such as may be developed with purified or semi-purified proteins or with lysates, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the in vitro system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or changes in enzymatic properties of the molecular target.

Administrations and Pharmaceutical Formulations

In certain embodiments, the present invention provides a method of treatment for a disease (disorder or condition) affected by aberrant activity of an mTOR-AP (e.g., GβL or rictor). Any disease that is responsive to mTOR-AP modulation can be treated by the method of the invention. Examples of such diseases include, but are not limited to, cancer, diabetes, and cardiovascular diseases (e.g., restenosis).

In an additional embodiment of the invention, an mTOR-AP polypeptide of the invention or fragment thereof is administered to an individual. For example, a rictor or a GβL polypeptide of the invention or fragment thereof is administered to an individual. In certain embodiments, both a rictor polypeptide or fragment thereof and a GβL polypeptide or fragment thereof are administered together to an individual. The individual can be a mammal such as a human.

When administered to an individual, the mTOR-AP polypeptide (e.g., rictor and/or GβL) can be administered as a pharmaceutical composition containing, for

example, the mTOR-AP polypeptide and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the mTOR-AP polypeptide (e.g., rictor and/or GβL). Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the composition.

One skilled in the art would know that a pharmaceutical composition containing an mTOR-AP polypeptide (e.g., rictor and/or GβL) can be administered to a subject by various routes including, for example, oral administration; intramuscular administration; intravenous administration; anal administration; vaginal administration; parenteral administration; nasal administration; intraperitoneal administration; subcutaneous administration and topical administration. The composition can be administered by injection or by incubation. The pharmaceutical composition also can be an mTOR-AP polypeptide (e.g., rictor and/or GβL) linked to a liposome or other polymer matrix. Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

EXEMPLIFICATION

Example 1. Identification of GβL as a subunit of the mTOR-signaling complex

Using cell lysis conditions that preserve the raptor-mTOR complex, Applicants isolated, from HEK-293T cells growing in nutrient-rich media, a 36-kDa protein that specifically interacts with mTOR. Coomasie-blue staining of SDS-PAGE analyses of mTOR immunoprecipitates revealed that the 36-kDa protein and mTOR are present in

near stoichiometric ratios (Fig. 1A). Raptor was found in substoichiometric amounts with mTOR (Fig. 1A). Mass spectrometric analysis identified the 36-kDa protein as GβL (G protein β subunit Like protein, pronounced 'Gable'), a widely expressed protein of unknown function (Rodgers, B. D., et al. (2001), J. Endocrinol *168*, 325-32). The structure of GβL consists almost entirely of seven WD40 repeats with high sequence similarity to those in the β subunits of heterotrimeric G-proteins (Rodgers, B. D., et al. (2001), J. Endocrinol *168*, 325-32) (Figures 17 and 18). Like mTOR and raptor, GβL is conserved among all eukaryotes, including *D. melanogaster, S. pombe, S. cerevisiae, C. elegans,* and *A. thaliana* (Ochotorena, I. L., et al. (2001), J. Cell Sci. *114*, 2911-20; Roberg, K. J., et al. (1997), Genetics *147*, 1569-84). Interestingly, genetic analyses show that Lst8p, the budding yeast homologue of GβL, regulates cell growth, the localization of amino-acid transporters and the expression of RTG genes, processes in which the TOR pathway plays a role (Roberg, K. J., et al. (1997), Genetics *147*, 1569-84; Liu, Z., et al. (2001), Embo. J. *20*, 7209-19). The fission yeast homologue of GβL, Wat1p, has functions in maintaining the stability of the genome and the integrity of microtubules (Ochotorena, I. L., et al. (2001), J. Cell Sci. *114*, 2911-20).

Example 2. GβL binds to the mTOR kinase domain specifically and independently or raptor

An anti-GβL antibody generated against residues 298-312 detected GβL in immunoprecipitates prepared from HEK-293T cells using an mTOR, but not control antibodies (Fig. 1B). Unlike raptor, GβL remained bound to mTOR in buffers containing the detergent Triton X-100, suggesting that the GβL-mTOR interaction does not require raptor. In addition to HEK-293T cells, Applicants also detected the mTOR-GβL complex in mouse NIH-3T3 and C2C12 cell lines. Furthermore, when over-expressed in HEK-293T cells, recombinant myc-tagged GβL interacted with endogenous (Fig. 1C) and co-expressed HA-mTOR (Fig. 1D).

To identify the GβL binding site(s) on mTOR, Applicants tested the capacity of full-length HA-GβL to interact with myc-tagged fragments of mTOR co-expressed in HEK-293T cells (Fig. 1E). GβL interacted strongly with the C-terminal half of mTOR (amino acids 1348 to 2549), a region that binds weakly, but specifically to raptor (Kim,

D. H., et al. (2002), Cell *110*, 163-75), On the other hand, GβL did not interact at all with the N-terminal half of mTOR (amino acids 1-1480), the region containing the principal binding site for raptor, the mTOR HEAT repeats. Further delineation of the GβL binding site revealed that GβL interacts with the mTOR kinase domain (amino acids 2115-2431) but not with the adjacent FRB domain (amino acids 2000-2115), the known binding site for FKBP12-rapamycin (Chen, J., et al. (1995), Proc. Natl. Acad. Sci. U.S.A. *92*, 4947-51). Truncation of the mTOR kinase domain destroyed its strong interaction with GβL, but revealed that two separate regions of mTOR, amino acids 2185-2254 and 2255-2431, weakly bind to GβL.

Example 3. GβL positively regulates the mTOR pathway

To determine the role of GβL in the mTOR pathway, Applicants investigated the effects on mTOR signaling of decreasing the expression level of GβL using siRNAs. Consistent with a critical role for GβL in mTOR function, a decrease in the expression of GβL reduced the in vivo phosphorylation state of S6K1 to a similar extent as a decrease in the expression of mTOR (Fig. 2A). The siRNA targeting GβL did not significantly affect the expression of S6K1 or ATM, or the phosphorylation state or amount of PKB1/Akt1, a downstream effector of PI 3-Kinase. Unlike raptor-targeting siRNAs (Kim, D. H., et al. (2002), Cell *110*, 163-75), siRNA targeting GβL did not reduce mTOR expression levels. The partial effects of the siRNAs targeting GβL and mTOR on S6K1 phosphorylation probably reflect a low transfection efficiency of the siRNAs. This was proven to be the case when Applicants monitored the activity of the mTOR pathway in individual cells using an immunofluorescence assay that detects phosphorylated S6 protein, a major S6K1 substrate. In HeLa cell monolayers transfected with siRNAs targeting mTOR, raptor, or GβL, but not lamin, many cells had no phospho-S6 staining, a result suggesting that these pathway components have necessary roles in mediating S6 phosphorylation (Fig. 2B). The mTOR pathway and S6K1 in particular, play a critical role in regulating cell growth and determining mammalian cell size (Fingar, D. C., et al. (2002), Genes Dev. *16*, 1472-87; Kim, D. H., et al. (2002), Cell *110*, 163-75). Consistent with a positive role for GβL in cell size control, actively growing HEK-293T cells transfected with siRNAs targeting GβL or mTOR underwent comparable reductions in

size compared to cells transfected with a control siRNA (Fig. 2C). Thus, these loss of function studies indicate that GβL has a positive, likely essential role in the mTOR pathway.

In testing potential mechanisms by which GβL exerts its positive function, Applicants found that co-expressing HA-GβL with myc-mTOR strongly increased the kinase activity of mTOR towards S6K1 and 4E-BP1 and its capacity for autophosphorylation (Fig. 2D). There was a dose-response relationship between the amount of co-expressed GβL and the level of mTOR activation. GβL-mediated stimulation of the mTOR kinase did not require raptor; as it occurred even when myc-mTOR was washed with buffers containing Triton X-100, conditions that removed endogenous raptor (Fig. 2E). These results suggest that stimulation of the mTOR kinase activity is an important mechanism by which GβL positively regulates the mTOR pathway.

Example 4. GβL mediates the interaction between raptor and mTOR

The stability of the GβL-mTOR association, unlike that of raptor-mTOR, was unaffected by nutrient conditions, such as leucine stimulation or deprivation, or by treatment with rapamycin or the mitochondrial inhibitor, antimycin A (Fig. 3A). In addition, the GβL-mTOR association was resistant to detergents like Triton X-100 (Fig. 1B) and Nonidet P-40 and high salt concentrations, suggesting that the interaction is not only constitutive, but also more stable than that of mTOR with raptor. An siRNA-mediated decrease in the expression level of GβL reduced the amount of both GβL and raptor bound to mTOR, implying that GβL has a role in stabilizing the raptor-mTOR interaction (Fig. 3B). Consistent with this, co-expression of HA-GβL with myc-mTOR significantly increased the amount of endogenous raptor that coimmunoprecipitates with myc-mTOR compared to when the latter was expressed alone (Fig. 3C).

To further explore how GβL stabilizes the association of raptor with mTOR, Applicants tested the possibility that GβL has independent binding sites for raptor and mTOR. They reasoned that if this were the case, they should be able to generate GβL mutants in which either the raptor or mTOR binding site is perturbed while the other is intact. Supporting this prediction, GβL point mutants m1, m4, and m6, when expressed

in HEK-293T cells, associated with endogenous mTOR as well as wild-type GβL, but interacted very weakly with endogenous raptor (Fig. 3D). On the other hand, Applicants could not generate GβL mutants that interact only with raptor and not mTOR, although mutants m2, m5, and m7 failed to interact with either protein. This result implies that for GβL to form a stable association with raptor it must also contact mTOR. Thus, in addition to the previously discovered association between raptor and the mTOR HEAT repeats (Kim, D. H., et al. (2002), Cell *110*, 163-75), raptor may also form an interaction with a structure consisting of GβL docked to the mTOR kinase domain. Supporting this possibility, an mTOR fragment consisting of mostly the kinase domain (amino acids 2115-2549) interacted with endogenous raptor only when it was co-expressed with wild-type GβL, but not the m1, m4, and m6 mutants (Fig. 3E). These results indicate that mTOR, raptor, and GβL form a heterotrimeric complex in which raptor interacts with both the mTOR HEAT repeats and a structure consisting of the GβL docked to the mTOR kinase domain. Because all the GβL mutants that bind mTOR also stimulated its in vitro kinase activity (Fig. 3F), the activating capacity of GβL is likely independent of its capacity to mediate the association of raptor with the mTOR.

Example 5. GβL forms part of the nutrient-sensitive interaction site for raptor on mTOR-GβL

Before the discovery of GβL, Applicants hypothesized that both a constitutive and a nutrient-sensitive interaction site mediates the association between raptor and mTOR (Kim, D. H., et al. (2002), Cell *110*, 163-75). They proposed that the nutrient-sensitive interaction strengthens under nutrient-poor conditions, stabilizes the mTOR-raptor complex so that it survives in vitro isolation, and leads to an inhibition of the mTOR kinase activity. Because GβL mediates the interaction between raptor and the mTOR kinase domain and stabilizes the raptor-mTOR interaction, Applicants considered the possibility that GβL is part of the nutrient-sensitive interaction site and that the GβL-raptor interaction is needed for raptor to inhibit the mTOR kinase activity. If this model is correct, GβL mutants that cannot target raptor to the mTOR kinase domain should protect mTOR from the inhibitory effects of raptor. Based on this model, Applicants would expect, then, that expression of these mutants would activate the mTOR parthway

in vivo. As predicted, expression of the GβL mutants (m1 or m4) with selective perturbations in the raptor-GβL (Fig. 4A) led to higher phosphorylation levels of co-expressed myc-S6K1 than did expression of wild-type GβL. Applicants next asked if the expression of these mutants could prevent the inhibitory effects of overexpressing wild-type raptor on the mTOR pathway (Kim, D. H., et al. (2002), Cell *110*, 163-75). The over-expression of wild-type raptor, but not of a mutant (mutant 9) that cannot bind mTOR (Kim, D. H., et al. (2002), Cell *110*, 163-75), decreased the phosphorylation state of a myc-S6K1 reporter. This effect was not blocked by the co-expression of wild-type GβL (Fig. 4B), but by the expression of the GβL mutants (m1 or m4) that cannot interact with raptor. Finally, as predicted, the m1 and m4 GβL mutants mimicked the destabilizing effects of nutrient-rich conditions on the association between raptor and the mTOR-GβL complex. Mutant-containing mTOR- GβL complexes bound less endogenous raptor than complexes with wild-type GβL (Fig. 3D and 4C). However, when cells were lysed in the presence of the cross-linker DSP, similar amounts of raptor were recovered with the mTOR-GβL complexes, irrespective of whether they contained wild-type or mutant GβL (Fig. 4C). Applicants have previously shown that DSP can similarly preserve the unstable interaction between raptor and mTOR seen under nutrient-rich conditions (Kim, D. H., et al. (2002), Cell *110*, 163-75). These observations indicate that, like nutrient-rich conditions, the GβL m1 and m4 mutants destabilize, but do not dissociate within cells, the association between raptor and mTOR-GβL. Thus, the raptor-GβL interaction site has the characteristics of the nutrient-sensitive interaction site and GβL is likely to play a critical role in mediating raptor regulation of mTOR activity. Furthermore, these results suggest that for raptor to inhibit the mTOR pathway it must interact with mTOR bound to GβL.

Consistent with this notion, in the absence of GβL, HA-raptor had only a small inhibitory effect on the kinase activity of co-expressed myc-mTOR. On the other hand, when the three proteins were expressed together to form a heterotrimeric complex, HA-raptor almost completely inhibited the HA-GβL-stimulated increase in myc-mTOR kinase activity (Fig. 4D). The raptor mutant 9 incapable of interacting with mTOR did not affect the basal or the GβL-stimulated mTOR kinase activity.

Results of work described herein indicate that inhibition of the mTOR kinase by raptor requires GβL and suggests a model in which the opposing actions of GβL and raptor regulate mTOR activity (Fig. 4E). They also support the conclusion that GβL interacts constitutively with mTOR, activates the mTOR kinase, and creates a nutrient-sensitive binding site for raptor. How GβL stimulates the mTOR kinase activity is unknown. It could contribute to the stability or folding of the mTOR kinase domain, a possibility supported by the finding that several heat shock protein bind the GβL-binding fragment of mTOR when it is expressed in the absence of GβL. GβL may also play a role in the recognition of mTOR substrates, although Applicants did not detect an interaction between GβL and S6K1 or 4E-BP1. Alternatively, GβL might recruit another, currently unidentified, protein that positively regulates mTOR function. Irrespective of the mechanism by which GβL activates mTOR, in Applicants' model the binding of raptor to the complex of GβL and the mTOR kinase domain inhibits this activation (Fig. 4E). Although the docking site for FKBP12-rapamycin, the FRB domain (Chen, J., et al. (1995), Proc. Natl. Acad. Sci. U.S.A. *92*, 4947-51), is directly N-terminal on mTOR to the GβL binding site, rapamycin does not significantly affect the amount of GβL bound to mTOR (Fig. 3A). Instead, rapamycin destabilizes the GβL-dependent interaction between mTOR and raptor (Kim, D. H., et al. (2002), Cell *110*, 163-75), and may inhibit mTOR activity by affecting the positive function of GβL.

Between GβL and raptor, the mTOR signaling complex contains 14 WD-40 repeats, which are found in numerous proteins involved in diverse cellular processes, including signal transduction, cell cycle progression, vesicular trafficking, and RNA processing (Neer, E. J., et al. (1994), Nature *371*, 297-300; Smith, T. F., et al. (1999), Trends Biochem. Sci. *24*, 181-5). In many cases, proteins containing WD-40 repeats form multimeric complexes with other proteins, in which the repeats serve as scaffolds for building the complexes (Smith, T. F., et al. (1999), Trends Biochem. Sci. *24*, 181-5). In a few cases, domains containing WD-40 repeats play a role in recruiting phosphorylated proteins to the catalytic sites of enzymes (Nash, P., et al. (2001), Nature *414*, 514-21; Yaffe, M. B. and Elia, A. E. (2001), Curr. Opin. Cell Biol. 13, 131-8). Interestingly, WD-40 repeat domains are also found in proteins interacting with PP2A, a phosphatase for which there is substantial evidence suggesting that it has a major role in

regulating downstream signaling by the mTOR pathway (Moreno, C. S., et al. (2002), J. Biol. Chem. *275*, 5257-63; Peterson, R. T., et al. (1999), Proc. Natl. Acad. Sci. U.S.A. *96*, 4438-42). Either as scaffolds or adaptors for recruiting substrates, the WD-40 repeat domains of GβL and raptor are likely to play important roles in regulation the mTOR pathway. Furthermore, as the kinase domain of mTOR, which is also the GβL-docking site, is fairly well-conserved amongst all the PIK-related proteins (Keith, C. T. and Schreiber, S. L. (1995), Science *270*, 50-1), it will be interesting to see whether other members of this family also bind GβL or related proteins.

Studies in fission yeast already hint that GβL is likely to participate in other signaling systems besides the TOR pathway. Mutations in Wat1p, the fission yeast homologue of GβL, lead to genomic instability and cell morphological changes, phenotypes not necessarily associated with TOR pathway dysfunction (Kemp, J. T., et al. (1997), Mol. Gen. Genset *254*, 127-38; Ochotorena, I. L., et al. (2001), J. Cell Sci. *114*, 2911-20). Furthermore, Wat1p interacts with Prp2p, the large subunit of the essential splicing factor U2AF (Ochotorena, I. L., et al. (2001), J. Cell Sci. *114*, 2911-20). Nevertheless, the function experiments described herein strongly indicate that GβL plays an essential, positive role in controlling cell growth by activating the mTOR kinase (Fig. 2A-2C).

The opposing effects on mTOR activity of the interactions mediated by GβL and raptor provide a mechanism by which cellular conditions, such as nutrient levels, can positively and negatively regulate mTOR signaling to the cell growth machinery. The balance between the actions of the two regulators may be perturbed in human diseases, such as cancer and diabetes, and could be artificially manipulated for potentially therapeutic benefits.

The following experimental procedures were followed in the work described herein.

Materials

Reagents were obtained from the following sources: DSP and Protein G-Sepharose from Pierce; ATP-{γ-^{32}P} from NEN; mTOR, S6K1, lamin, and Rho antibodies as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary

antibodies from Santa Cruz Biotechnology; Phospho-T389 S6K1 and Phospho-S473 PKB/ Akt antibodies from Cell Signaling; HA monoclonal antibody from Covance; myc monoclonal antibody from Oncogene Research Products; myc rabbit polyclonal antibody from Upstate Biotechnology; DMEM, leucine, glucose, RPMI, and RPMI without leucine from Life Technologies; and rapamycin, FK506, valinomycin, antimycin A, and 2-deoxyglucose from Calbiochem. The rabbit polyclonal anti-GβL antibody, recognizing residues 298-312 of human GβL, was produced using the custom antibody service from Covance.

Purification and Identification of GβL

mTOR immunoprecipitates prepared from 200 million HEK293T cells were prepared as above, resolved by SDS-PAGE, and proteins visualized by Coomassie blue staining. The band corresponding to GβL was excised and trypsinized as described (Erdjument-Bromage et al., 1994). A hundred percent of the generated peptides were subjected to a micro-clean-up procedure using 2 μL bed-volume of Poros 50 R2 (PerSeptive) reversed-phase beads packed in an Eppendorf gel-loading tip. Mass Spectrometry (Maldi-ReTOF was then carried out on two peptide pools (16 and 30% MeCN) recovered from the RP-microtip column using a Bruker REFLEX III instrument with delayed extraction. For mass fingerprinting, top major experimental masses (*m/z*) combined from both MALDI-ReTOF experiments were used to search a non-redundant human protein database (NR; ~66,605 entries; NCBI; Bethesda, MD), using the PeptideSearch (M. Mann, University of Southern Denmark) algorithm. A molecular weight range twice the predicted weight was covered with a mass accuracy restriction better than 40 ppm, and maximum one missed cleavage site was allowed per peptide. Alternatively mass spectrometric-based sequencing (ESI-MS/MS) of selected peptides from partially fractioned pools was carried out using a PE-SCIEX API300 triple Quadrupole instrument, fitted with a continuous flow nano-electrospray source (JaFIS). All peptides masses in pools were obtained by DE-MALDI-reTOF MS (BRUKER Reflex III). Peptide Sequences were obtained by nono-electrospray tandem MS (JaFIS@ source with SCIEX ApI300 triple quadrupole).

Immunoprecipitations

10 X 10^6 HEK-293T cells growing in 10 cm dishes were rinsed once with PBS and lysed in 1 ml of ice-cold Buffer B (40 mM Hepes pH 7.5, 120 mM Nacl, 1 mM EDTA, 10 mM glycerphosphate, 50mM NaF, 1.5 mM $Na_3 VO_4$ 0.3% CHAPS, and one tablet EDTA-free protease inhibitors (Roche) per 10 ml). After clearing the lysates by centrifugation at 10,000Xg for 10 min, 30 µl of a 50% slurry of protein G-Sepharose and 4 µg of the immunoprecipitating antibody was added to the supernatant. After a 3-hour incubation at 4 °C, immunoprecipitates were washed four times with Buffer B and once with Wash Buffer 1 (50 mM Hepes pH 7.5, 40 mM NaCl, and 2 mM ETDA). Samples were resolved by SDS-PAGE, proteins transferred to PVDF and used for immunoblotting as described (Burnett, P. E., et al. (1998), PNAS *95*, 1432-1437). When Triton X-100 was used to eliminate raptor binding to a mTOR, immunoprecipitates were prepared as above except that Buffer A (Buffer B with 1% Triton X-100 instead of CHAPS) was used to lyse the cells.

Cloning of the GβL cDNA, DNA Manipulations and Mutagenesis

The human GβL clone was obtained from Incyte (clone ID 3C6), subcloned into myc- and HA-prk5 vectors by PCR, and transfected into HEK-293T cells using lipofectamine 2000 as described by the manufacturer (Invitrogen). The mTOR fragments indicated in Figures 1 and 19 were expressed from cDNA's subcloned into the myc-prk5 vectors. The GβL open reading frame in pBluescript II SK (+) was mutagenized using the QuickChange mutagenesis kit (Stratagene) as described by the manufacturer and subcloned into the SalI and NotI sites of myc- and HA-prk5. The GβL mutants used in this study are: m1 (D42A); m2 (S72D); m4 (A182D); m6 (T208D); m7 (F320S). The mutated sites are indicated in the alignment of the WD40 repeats (Figure 17) and the structural model of GβL (Figure 18). All other epitope tagged constructs have been described (Burnett, P. E., et al. (1998), PNAS *95*, 1432-1437; Kim, D. H., et al. (2002), Cell *110*, 163-75).

Plasmid and siRNA Transfections

3 million HEK293T cells in 6-cm dishes were transfected with plasmid constructs indicated in the Figure legends using the Lipofectamine 2000 transfection reagent (Life Technology). 24 hours after DNA addition, cells were rinsed once with PBS and lysed in 300 µl of ice cold Buffer B. Immune complexes were prepared from cleared supernatants using 3 µg polyclonal anti-myc or monoclonal anti-HA antibodies and 20 µl of a 50% slurry protein G-Sepharose. After a 3-hour incubation, immuneprecipitates were washed six times with Buffer B and twice with Wash Buffer 2. Bound proteins were eluted in 1X sample buffer, and mTOR or HA- or myc-tagged proteins were detected by immunoblotting as described (Burnett, P. E., et al. (1998), PNAS *95*, 1432-1437). 21-nucleotide complementary RNAs with 2-nucleotide overhangs (Elbashir, S. M., et al. (2001), Nature *411*, 494-8) were designed to target bases 188-210 if the GβL open reading frame. The sequences for the siRNAs for lamin, mTOR, and raptor, the transfection conditions and the procedures for determining cell size have been described (Kim, D. H., et al. (2002), Cell *110*, 163-75).

Immunofluorescence

HeLa cells transfected with siRNA targeting lamin, GβL, mTOR, or raptor were harvested one day after transfection and seeded onto 1.5-cm diameter gelatin-coated glass coverslips. 48 hrs after seeding, the were fixed in a 3.7% paraformaldehyde for 20 min at room temperature, washed twice with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After washing twice with PBS and blocking with 1% BSA for 1 hr, the cells were incubated overnight with an anti-phospho S6 antibody (Cell Signaling Tech.). The cells were then washed twice with PBS, incubated with anti-rabbit cy3 (Jackson Immunolabs) and Hoechst for 30 min, washed with PBS, mounted in glycerol containing 0.1% p-phenylenediamine and visualized with fluorescence microscopy.

Sequence Alignments and Model Building

The WD40 repeat sequences of GβL were aligned with ClustalX v1.81 (Thompson, J. D., et al. (1997), Nucleic Acids Res. *25*, 4786-82) using the Gonnet series weight matrix. Pairwise gap opening and gap extension penalties were set at 10.00 and 0.10 respectively. Multiple alignment gap opening and gap extension penalties were set

at 10.00 and 0.20. The GβL model was built and optimized with Modeler (Sali, A., and Blundell, T. L. (1993), J. Mol. Biol. *234*, 779-815) using the coordinates of the TUP1 β chain (pdb: 1ERJ accession: 1ERJ_B) as the template.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

Materials

Reagents were obtained from the following sources: DSP and Protein G-Sepharose from Pierce; ATP-{γ-^{32}P} from NEN; mTOR, S6K1, lamin, and Rho antibodies as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; Phospho-T389 S6K1 and Phospho-S473 PKB/ Akt antibodies from Cell Signaling; HA monoclonal antibody from Covance; myc monoclonal antibody from Oncogene Research Products; myc rabbit polyclonal antibody from Upstate Biotechnology; DMEM, leucine, glucose, RPMI, and RPMI without leucine from Life Technologies; and rapamycin, FK506, valinomycin, antimycin A, and 2-deoxyglucose from Calbiochem. The rabbit polyclonal anti-GβL antibody, recognizing residues 298-312 of human GβL, was produced using the custom antibody service from Covance.

Example 6. Another novel binding partner of mTOR, rictor, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton.

Growth is an energetically costly cellular process that is exquisitely regulated by environmental and cellular conditions. Increasing evidence indicates that mTOR is the central component of a pathway that regulates growth in mammals in response to nutrients, growth factors and stress. This protein is also the target of the complex formed by the macrolide rapamycin and its receptor FKBP12. Like a reduction in mTOR

expression, rapamycin causes a decrease in cell size and inhibits all known downstream effectors of mTOR (Gingras, et al., 2001, Prog Mol Subcell Biol, 27, 143-74; Kozma & Thomas, 2002, Bioessays, 24, 65-71; Schmelzle & Hall, 2000, Cell, 103, 253-62). When given to mice in utero the drug also phenocopies an embryonic lethal allele of mTOR (Hentges, et al., 2001, Proc Natl Acad Sci USA, 98, 13796-801). Thus, rapamycin seems to cause a loss of mTOR function and is often used to determine if mTOR participates in a cellular or molecular process of interest. Rapamycin is also an important drug with several current and potential clinical uses, including immunosuppression (Saunders, et al., 2001, Kidney Int, 59, 3-16), prevention of cardiac vessel restenosis (Morice, et al., 2002, N Engl J Med, 346, 1773-80; Sousa, et al., 2001, Circulation, 103, 192-195), anti-cancer therapy (Vogt, 2001, Trends Mol Med, 7, 482-4; Podsypanina, et al., 2001, Proc Natl Acad Sci USA, 98, 10320-5; Neshat, et al., 2001, Proc Natl Acad Sci USA, 98, 10314-9) and treatment of the genetic syndrome tuberous sclerosis complex (Gao, et al., 2002, Nat Cell Biol, 4, 699-704; Inoki, et al., 2002, Nat Cell Biol, 12, 12; Jaeschke, et al., 2002, J Cell Biol, 159, 217-24; Kwiatkowski, et al., 2002, Hum Mol Genet, 11, 525-34; Tee, et al., 2002, Proc Natl Acad Sci USA, 99, 13571-6; Kenerson, et al., 2002, Cancer Res, 62, 5645-50). mTOR does not act alone as it associates with at least two other proteins, raptor and GβL (Kim, et al., 2002, Cell, 110, 163-175; Kim, et al., 2002, Molecular Cell, 11, 895-904; Hara, et al., 2002, Cell, 110, 177-89). Both of these proteins regulate cell size (Kim, et al., 2002, Cell, 110, 163-175; Kim, et al., 2002, Molecular Cell, 11, 895-904), and are conserved in all eukaryotic model organisms (Kim, et al., 2002, Cell, 110, 163-175; Kim, et al., 2002, Molecular Cell, 11, 895-904; Hara, et al., 2002, Cell, 110, 177-89; Loewith, et al., 2002, Mol Cell, 10, 457-68; Wedaman, et al., 2003, Mol Biol Cell, 14, 1204-20; Roberg, et al., 1997, Genetics, 147, 1569-84). Raptor has multiple functions in the mTOR complex, serving as an adaptor protein for mTOR substrates (Hara, et al., 2002, Cell, 110, 177-89; Nojima, H. et al., 2003, J Biol Chem, 278, 15461-4; Choi, et al., 2003, J Biol Chem, 278, 19667-73; Schalm, et al., 2003, Curr Biol, 13, 797-806) as well as regulating mTOR activity in response to nutrients (Kim, et al., 2002, Cell, 110, 163-175). Here, Applicants identified a novel mTOR-binding protein called rictor for the reasons described below. The rictor-containing mTOR complex is distinct from the one containing raptor and does not regulate cell size or known mTOR

effectors and is not a target of rapamycin. Instead, Applicants found that rictor and mTOR, but not raptor, regulate a distinct pathway that modulates the phosphorylation state of Protein Kinase C alpha (PKCα) and the organization of the actin cytoskeleton.

To identify novel components of the mTOR signaling complex, Applicants purified mTOR with methods that preserve the raptor-mTOR interaction (Kim, et al., 2002, Cell, 110, 163-175). In earlier work, Applicants have shown that the interaction between raptor and mTOR is unstable in buffers containing certain detergents, such as Triton X-100, but preserved in others such as CHAPS. Applicants had previously noticed that the mTOR complex immunopurified from HEK293T cells contains a low abundance 200 kDa protein, but only when purified from HeLa cells did the complex contain enough of this protein for its identification (Fig. 11A). Like the mTOR-raptor interaction, the mTOR-p200 interaction is sensitive to Triton X-100, but stable in CHAPS-containing buffers (Fig. 11A).

Peptide mass fingerprinting analysis using matrix-assisted laser desorption/ ionization-time-of-flight (MALDI-TOF) mass spectrometry and mass spectrometric sequencing revealed that p200 is novel and not represented in the databases of full-length human proteins. Applicants named the 200 kDa protein rictor for rapamycin insensitive companion of mTOR. Starting from a truncated cDNA that encodes part of rictor (accession # KIAA1999), Applicants used EST mining and RT-PCR to assemble a full-length rictor open reading frame that predicts a protein of 1708 amino acids with a molecular weight of 192 kDa. Applicants could not identify any domains of known function in rictor and, compared to mTOR, raptor and GβL, the protein is not well conserved amongst eukaryotes. Rictor shares regions of homology with several poorly characterized proteins, including pianissimo from D. discoidieum (Chen, et al., 1997, Genes Dev, 11, 3218-31), Ste20p from S. pombe (Hilti, et al., 1999, Curr Genet, 35, 585-92, and Avo3p from S. cerevisiae). In addition, proteins of unknown function but of similar domain structure and conservation are encoded in the A. gambiae and D. melanogaster genomes, suggesting that most eukaryotes may have rictor-like proteins. These proteins share a region of about 200 amino acids in length (box 1 in Fig. 11B) of 44% similarity (8% identity) as well as several smaller conserved regions, including a repeated block of 20 amino acids (box 5 in Fig. 11B). Despite these regions of similarity

both rictor and dRictor, its likely Drosophila homologue, have long c-terminal extensions without any apparent conservation and with no similarity to other proteins. Because of its poor conservation with pianissimo, Ste20p, and Avo3p, it is not surprising that rictor was not identified using approaches based on sequence similarity. Pianissimo is implicated in cAMP-induced cell migration (Chen, et al., 1997, Genes Dev, 11, 3218-31) and in S. cerevisiae Avo3p is part of a TOR2p-containing complex that regulates the actin cytoskeleton (Loewith, et al., 2002, Mol Cell, 10, 457-68). Moreover, earlier work in S. pombe shows that ste20p is needed for cell cycle arrest in response to nutrient deprivation (Hilti, et al., 1999, Curr Genet, 35, 585-92).

Using the antibody that recognizes human rictor, Applicants confirmed that rictor is part of the endogenous mTOR complex and does not co-immunoprecipitate with a control protein (Fig. 11C). Nutrient levels and mitochondrial function regulate the activity of S6K1 and 4E-BP1 (Kim, et al., 2002, Cell, 110, 163-175; Hara, et al., 1998, J Biol Chem, 273, 14484-94; Lynch, et al.,2000, J Cell Biochem, 77, 234-51; Dennis, et al. 2001, Science, 294, 1102-5; Xu, et al., 2001, Diabetes, 50, 353-60) as well as the stability of the raptor-mTOR interaction (Kim, et al., 2002, Cell, 110, 163-175), but these conditions do not affect the mTOR-rictor interaction. In particular, leucine levels and electron transport inhibition by antimycin A modulate the raptor- but not rictor-mTOR association (Fig. 11C). Similarly, under the appropriate cell lysis conditions, rapamycin treatment of cells eliminates the binding of mTOR to raptor (Kim, et al., 2002, Cell, 110, 163-175) without affecting the interaction of rictor with mTOR (Fig. 11C). Like raptor but not the control protein GCP3, overexpressed recombinant myc-rictor enters the endogenous mTOR complex and recombinant rictor can be used to immunoprecipitate endogenous mTOR (Fig. 11D). The interaction between recombinant rictor and mTOR, like that between the endogenous proteins, is stable in CHAPS-containing buffers but sensitive to Triton X-100 (Fig. 11D).

In mTOR complexes isolated from HEK293T cells raptor and mTOR are in a nearly stoichiometric ratio (0.8-0.9 raptor to 1.0 of mTOR) (Kim, et al., 2002, Cell, 110, 163-175). During the purification of rictor it became apparent that in HeLa cells this is not the case, and that complexes in these cells have less raptor but more rictor. When comparing mTOR complexes across mammalian cell types, Applicants always observed

an inverse correlation between the amounts of raptor and rictor (Fig. 12A). For example, complexes in HeLa, HEK293T, and DU145 cells contain about the same amount of mTOR but in HeLa and DU145 cells the complexes have more rictor than raptor while the opposite is true in HEK293T cells (Fig. 12A). To determine if mTOR is in distinct complexes within cells, Applicants isolated the complex from HEK293T cells using antibodies recognizing mTOR, raptor or rictor and then determined the composition of the isolated complexes. As expected, mTOR isolated with the mTOR antibody associates with raptor, rictor and GβL in a detergent-sensitive manner (Fig. 12B). On the other hand, complexes isolated with the raptor antibody contain mTOR and GβL but not rictor while those isolated with the rictor antibody contain mTOR and GβL but not raptor (Fig. 12B). Thus, it appears that raptor and rictor independently associate with mTOR and GβL, defining two distinct mTOR complexes—one containing raptor and the other rictor. In accordance with this possibility, the expression of wild-type raptor, but not of a mutant that cannot bind mTOR (Kim, et al., 2002, Cell, 110, 163-175), strongly suppresses the interaction of co-expressed rictor with mTOR (Fig. 12C). These findings provide evidence that mammalian cells contain at least two distinct mTOR-containing complexes, reminiscent of recent work (Loewith, et al., 2002, Mol Cell, 10, 457-68; Wedaman, et al., 2003, Mol Biol Cell, 14, 1204-20), indicating that this is the case for TOR2p in budding yeast.

As rapamycin did not affect the interaction between rictor and mTOR (Fig. 11C), Applicants asked if the rictor-containing complex interacts with FKBP12-rapamycin and participates in rapamycin-sensitive processes. Our previous work shows that the binding of FKBP12-rapamycin to the raptor-containing complex destabilizes the raptor-mTOR interaction so that no raptor remains bound to immunopurified mTOR. As other groups have not observed this (Hara, et al., 2002, Cell, 110, 177-89; Loewith, et al., 2002, Mol Cell, 10, 457-68), Applicants suspected that a component of our buffer system might be critical for the destabilizing effect of rapamycin on the mTOR-raptor interaction. This turns out to be the case—Applicants find that for rapamycin to affect the interaction the cell lysis buffer must contain a molecule with a phosphate group (sodium pyrophosphate and beta-glycerophosphate in our buffer) (Fig. 13A). Using a phosphate-free buffer, Applicants were able to show that, in the presence of rapamycin, HA-FKBP12 expressed

in HEK293T cells binds to the raptor- but not the rictor-containing mTOR complexes (Fig. 13B). This finding suggests that rictor is unlikely to participate in mTOR functions discovered through the use of rapamycin.

To confirm this notion, Applicants asked whether rictor plays a role in regulating the ribosomal S6 Kinase 1 (S6K1), a critical controller of cell size and an mTOR substrate (Burnett, et al., 1998, PNAS, 95, 1432-1437; Isotani, et al., 1999, J Biol Chem, 274, 34493-8) whose phosphorylation state is rapamycin-sensitive (Kuo, et al., 1992, Nature, 358, 70-73; Chung, et al., 1992, Cell, 69, 1227-1236; Price, et al., 1992, Science, 257, 973-7). Unlike reductions in raptor or mTOR expression (Kim, et al., 2002, Cell, 110, 163-175), siRNA-mediated knock-down of rictor levels do not decrease the phosphorylation of S6K1 in either HEK293T or HeLa cells. In contrast, Applicants reproducibly observe a slight increase in phospho-S6K1 that correlates with a small increase in the amount of raptor in the mTOR complexes isolated from cells with reduced rictor expression (Fig. 13C). Similarly, in Drosophila S2 cells dsRNA-induced RNAi against dS6K, dTOR or dRaptor eliminates the phosphorylation of dS6K while a dsRNA targeting dRictor causes an increase in dS6K phosphorylation (Fig. 13D). As might be expected, the activation of S6K caused by reductions in rictor levels leads to a small increase in mean cell size in both human and Drosophila cells (data not shown). Thus, it appears that rictor is neither a positive regulator of cell size nor of S6K phosphorylation and that intracellularly the composition of the mTOR complex is dynamic so that decreases in rictor levels lead to increases in the amount of the raptor-containing mTOR complex.

Consistent with these findings, the rictor-containing mTOR complex purified from either HEK293T or HeLa cells does not phosphorylate S6K1 in vitro (Fig. 13E). In contrast, mTOR complexes that contain raptor— isolated with antibodies recognizing either mTOR or raptor— phosphorylate S6K1 in a rapamycin sensitive fashion. A potential explanation for the inability of the rictor-containing complex to phosphorylate S6K1 might be that when bound to rictor mTOR is inactive. This is unlikely to be true because mTOR still autophosphorylates in rictor-containing complexes from HeLa or HEK293T cells (see arrows in Fig. 13E). In addition, the rictor complex phosphorylates the non-physiological substrate myelin basic protein (MBP) more effectively than the

raptor complex (data not shown). Moreover, in kinase assays a protein of the same apparent molecular weight as rictor clearly becomes phosphorylated (see arrows in Fig. 13E), suggesting that rictor itself may be a substrate for the mTOR kinase activity when it is bound to mTOR. This appears to be the case because in cells metabolically labeled with radioactive phosphate a reduction in mTOR expression decreases the amount of radioactivity in rictor without affecting rictor expression (Fig. 16A). In addition, in cells with reduced mTOR expression rictor appears as a doublet in SDS-PAGE analyses, suggesting that dephosphorylated rictor migrates more quickly than the phosphorylated protein (Fig. 16B), a result Applicants confirmed using in vitro phosphatase treatment of immunoprecipitated rictor (Fig. 16C). Applicants used the phosphorylation-induced shift in rictor migration as a convenient way to search for conditions that affect rictor phosphorylation within cells. As expected for an mTOR-dependent phosphorylation, treatment of cells with LY294002, a PI 3-kinase inhibitor that also directly inhibits mTOR kinase activity (Brunn, et al., 1996, EMBO Journal, 15, 5256-5267), increased rictor mobility while rapamycin had no effect. Of the many different stress conditions, Applicants tested, only a sorbitol-induced osmotic stress also increased rictor mobility (Fig. 16D).

Only recently are Applicants beginning to understand the biochemical composition and regulation of the raptor-containing mTOR complex that is the target of rapamycin. Even before its characterization, many molecular (e.g., S6K1 phosphorylation) and cellular (e.g., cell size control) functions were ascribed to it because of their sensitivity to rapamycin. As the rictor-containing mTOR complex does not appear to participate in rapamycin-sensitive processes, there were no leads for identifying effectors downstream of rictor and insight into a function for rictor came from a fortuitous observation. In immunoblots prepared from cells with reduced rictor expression, Applicants noticed a decrease in the intensity of a faint background band recognized by the phospo-T389 S6K1 antibody. Applicants reasoned that the antibody must be cross-reacting with a protein containing a similar phosphorylation site. Through motif searching Applicants identified several candidate Protein Kinase C (PKC) isoforms with phosphorylation sites that are similar to T389 of S6K1. By testing phosphospecific antibodies that recognize these sites, Applicants discovered that a reduction in rictor

expression leads to a specific decrease in the phosphorylation of S657 of PKCα. In HeLa cells the S657 phosphospecific antibody is specific for PKCα as an siRNA-mediated reduction in PKCα reduced the intensity of the band recognized by the antibody on a western blot (Fig. 14A). Using lentiviral-mediated expression of siRNAs (Stewart, et al., 2003, RNA, 9, 493-501), Applicants generated a set of HeLa cell lines with substantially reduced levels of rictor, raptor or mTOR (Fig. 14B). As expected, reductions in raptor or mTOR expression or rapamycin treatment greatly decreased the phosphorylation of S6K1 (Fig. 14B). On the other hand, a reduction in rictor expression slightly increased S6K1 phosphorylation while decreasing PKCα phosphorylation. Consistent with rictor and mTOR functioning together, a reduction in mTOR expression also decreased PKCα phosphorylation. The positive role of rictor-mTOR in mediating PKCα phosphorylation appears to be evolutionarily conserved as RNAi-mediated decreases in dRictor and dTOR, but not dRaptor, reduced, in western blots, the phosphorylation of a band with the predicted molecular weight of Drosophila PKCα (Fig. 14C). Phosphorylation of mammalian PKCα on the rictor- and mTOR-dependent site is absolutely necessary for its kinase activity (Hansra, et al., 1999, Biochem J, 342 (Pt 2), 337-44; Bornancin & Parker, 1996, Curr Biol, 6, 1114-23), suggesting that the mTOR-rictor complex has a critical role in regulating the activity of this kinase. The mTOR- and rictor-dependent phosphorylation of PKCα represents the first marker of activity for the rictor-containing mTOR complex. In this regard it is similar to the identification, more than a decade ago, of the rapamycin-sensitive phosphorylation of S6K1 (Kuo, et al., 1992, Nature, 358, 70-73; Chung, et al., 1992, Cell, 69, 1227-1236; Price, et al., 1992, Science, 257, 973-7), that Applicants now recognize as the first biochemical marker for the activity of the raptor-containing mTOR complex.

In mammalian cells, PKCα is ubiquitously expressed and has been implicated in a large number of varied cellular processes, including apoptosis, growth, cell cycle control and the regulation of cell shape and mobility (Bornancin & Parker, 1996, Curr Biol, 6, 1114-23). The HeLa cell lines with constitutively reduced levels of rictor do not have any apparent defects in cell proliferation but Applicants noticed through visual inspection that these cells were flatter and had a more square-like shape than controls. Because PKCα

controls the actin cytoskeleton in some cell types (Hai, et al., 2002, Exp Cell Res, 280, 64-74) and in yeast the TOR proteins have a rapamycin-insensitive function in regulating actin organization (Loewith, et al., 2002, Mol Cell, 10, 457-68; Schmidt, et al., 1996, Proc Natl Acad Sci USA, 93, 13780-5), Applicants reasoned that an altered actin cytoskeleton might account for the perturbed morphology of the rictor knockdown cells. To examine this possibility Applicants used fluorophore-tagged phalloidin to stain for actin in cells with constitutively reduced levels of rictor, mTOR or raptor (Fig. 15A, B). In control cells, actin localizes to the cell cortex as well as diffusely and weakly throughout the cell cytoplasm (Fig. 15A). Cells with reduced raptor expression are smaller than controls but otherwise have a similar pattern of actin localization that features prominent cortical staining (Fig. 15A). In contrast, cells with reduced levels of rictor show a dramatically altered pattern of actin localization. In these cells, thick disorganized actin fibers are present throughout much of the cytoplasm and cortical actin is far less prominent. Many cells have cytoplasmic bundles of thick actin fibers without clear connections to the remainder of the actin cytoskeleton (arrow in Fig. 15A, rictor actin panel). Consistent with rictor and mTOR functioning together, in cells with reduced mTOR expression, the pattern of the actin staining is similar to that in rictor knockdown cells. Although more difficult to appreciate because of the greatly reduced size of mTOR knockdown cells, these cells also have cytoplasmic bundles of thick actin fibers (arrow in Fig. 15A, mTOR actin panel). The localization of paxillin, an adaptor protein present at the junction between the actin cytoskeleton and the plasma membrane (Turner, 2000, Nat Cell Biol, 2, E231-6), also reveals the altered organization of the actin cytoskeleton in the rictor and mTOR knockdown cells. These cells have many cytoplasmic paxillin patches that colocalize to the ends of thick actin fibers while in the control and the raptor knockdown cells the paxillin patches are present mainly at the cell periphery within cellular extensions (Fig. 15A, B). As Applicants have identified PKCα as downstream of rictor-mTOR, Applicants asked if PKCα also regulates the actin cytoskeleton in HeLa cells (Fig. 15C). The morphology of the actin cytoskeleton in cells with siRNA-mediated reductions in PKCα is reminiscent but not a complete mimic of that in the rictor knockdown cells. Both show thick cytoplasmic actin fibers and less cortical actin staining than controls, but in the PKCα knockdown cells the thick actin fibers appear more

numerous, and better organized and connected to the remainder of the cytoskeleton. Thus, our findings indicate that rictor and mTOR regulate the organization of the actin cytoskeleton and suggest that PKCα is an important mediator of this function. Evidence from other species supports our finding that rictor regulates PKCα and the actin cytoskeleton. In *Dictyostelium*, pianissimo, the rictor homologue, has a positive role in mediating cAMP-induced cell aggregation (Chen, et al., 1997, Genes Dev, 11, 3218-31), a process in which PKC-like kinases are thought to be involved (Phillips, et al., 1997, Biochim Biophys Acta, 1349, 72-80). The likely rictor homologue in budding yeast, Avo3p, forms part of a complex containing TOR2p that regulates—in a rapamycin-insensitive fashion— the actin cytoskeleton. As the raptor-mTOR complex controls cell size through at least two effectors—S6K1 and 4E-BP1 (Fingar, et al., 2002, Genes Dev, 16, 1472-87) — it is probable that the rictor-mTOR complex will have more than one effector involved in controlling the organization of the cytoskeleton.

Applicants have discovered a novel complex in mammalian cells that contains mTOR but not its previously identified partner protein raptor. Instead, rictor, a new protein of unknown function, defines this complex, and the proportion of mTOR within rictor- and raptor-containing complexes varies across mammalian cell types. Currently, Applicants can only speculate as to what the rictor branch of the mTOR pathway senses as in our preliminary data Applicants find no evidence that the signals that regulate the raptor part of the mTOR pathway, such as nutrients and growth factors, regulate PKCα phosphorylation or the mTOR-rictor association. Unlike the raptor complex, the one containing rictor does not appear to be bound nor inhibited by FKBP12-rapamycin and is thus unlikely to participate in most cellular functions ascribed to mTOR based on their sensitivity to rapamycin treatment. However, small molecules that directly inhibit the kinase activities of mTOR, such as the well-known PI 3-kinase inhibitor, LY294002, appear to suppress the kinase activity of mTOR in the rictor complex. Thus, it is possible that the rictor complex mediates functions assigned to PI 3-Kinase because of their sensitivity to LY294002 and insensitivity to rapamycin. Now that two distinct mTOR complexes are known, with different downstream targets, it may be possible to isolate small molecules that selectively inhibit the rictor branch of the pathway which, in turn, are likely to have different pharmacological effects than rapamycin and direct mTOR

inhibitors. Applicants have identified the phosphorylation of PKCα and the organization of the actin cytoskeleton as molecular events downstream of the rictor-mTOR complex. This finding is a critical step towards dissecting the signaling pathways controlled by the two distinct mTOR complexes and hints at the previously unrecognized complexity of the TOR network in mammalian systems.

Methods and Materials

Reagents were obtained from the following sources: protein G-sepharose from Pierce; ATP-[γ-^{32}P] from NEN; mTOR, S6K1, and PKCα antibodies as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; phospho-T389 S6K1 and phospho-PKCα/β_2 antibodies from Cell Signaling; HA monoclonal antibody from Covance; myc monoclonal antibody from Oncogene Research Products; Drosophila S6K antibody from Mary Stewart, North Dakota State University; Alexa Fluor 488-conjugated secondary anti-mouse antibody and Texas Red-X-phalloidin from Molecular Probes; paxillin monoclonal antibody from BD Transduction Laboratories; DMEM, leucine, glucose, RPMI, and RPMI without leucine from Life Technologies; rapamycin, LY294002, and antimycin A from Calbiochem. The GßL antibody was described previously (Kim et al., 2002, Cell, 110:163-175), and the rictor and raptor antibodies were developed with the antibody service from Covance using the following peptides: (rictor: RGRSLKNLRVRGRND, amino acid sequence 6-20) and (raptor: mesemlqspllglgeedead, amino acid sequence 1-20).

Purification and identification of rictor

mTOR immunoprecipitates prepared from 60 million HeLa cells were resolved by SDS-PAGE, and proteins visualized by Coomassie blue or silver staining. The ~200 kDa band corresponding to rictor was digested with trypsin, the mixtures fractionated on a Poros 50 R2 RP micro-tip, and resulting peptide pools analyzed by matrix-assisted laser-desorption / ionization reflectron time-of-flight (MALDI-reTOF) MS using a BRUKER UltraFlex TOF/TOF instrument (Bruker Daltonics; Bremen, Germany), as described (Erdjument-Bromage, et al., 1998, J Chromatogr A, 826, 167-81; Sebastiaan Winkler, et al., 2002, Methods, 26, 260-9). Selected experimental masses (*m/z*) were taken to search

the human segment of a non-redundant protein database ('NR'; ~108,000 entries; National Center for Biotechnology Information; Bethesda, MD), utilizing the PeptideSearch (Matthias Mann, Southern Denmark University, Odense, Denmark) algorithm, with a mass accuracy restriction better than 40 ppm, and maximum one missed cleavage site allowed per peptide. Mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples, using the UltraFlex instrument in 'LIFT' mode. Fragment ion spectra were taken to search NR using the MASCOT MS/MS Ion Search program (Matrix Science Ltd.; London, UK). Any identification thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data.

Cloning of the full-length human rictor cDNA and its sequence analysis

Human and mouse cDNA and EST sequences obtained from public databases were used to electronically assemble a putative full-length cDNA. The human KIAA1999 cDNA was the largest fragment available but is missing ~1.3 kb of 5' coding sequence. To prepare the full-length rictor cDNA three DNA fragments were combined: a human EST (BG623200), a human cDNA (KIAA1999) and a PCR product spanning the gap between the EST and cDNA that was made from 1st strand cDNA derived from HeLa cell total RNA. The PCR product was prepared using a forward primer corresponding to a sequence 5' of the BamH1 site at position 950 of the rictor ORF and a reverse primer corresponding to a sequence 3' of the PacI site at position 1616 and was added to the 3' end of the BG623200 EST. The SalI/PacI and PacI/XmaI segments of the extended BG623200 and KIAA1999, respectively, were subcloned into the prk5 expression vector in a three-way ligation. Prior to use, a corrupted section between the SpeI sites at 2682 and 3196 of the KIAA1999 cDNA was replaced with a wild-type fragment obtained by RT-PCR. In order to make these SpeI sites unique for the repair, the third SpeI site at 9135 of KIA1999 was removed by excising the non-coding fragment between SwaI sites at 6934 and 9367. All rictor fragments generated by PCR were confirmed by DNA sequencing.

Rictor sequences from several species were analyzed using the MEME Motif Discovery Tool (Bailey & Elkan, 1994, Proc Int Conf Intell Syst Mol Biol, 2, 28-36) to

identify regions of sequence conservation and internal repeats. A motif length range of 20-50 amino acids was imposed on the algorithms. One internal repeat was found among all of the analyzed sequences. The repeats (except for S. cerevisiae) were aligned to each other using Clustalx v.1.81 (Thompson, et al., 1997, Nucleic Acids Res, 25, 4876-82).

Immunoprecipitations, kinase assays, and metabolic labeling

3 X 10^6 HeLa or HEK293T cells growing in 10-cm dishes were rinsed once with cold PBS and lysed on ice for 20 min in 1 ml of ice-cold Lysis Buffer (40 mM Hepes pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, and EDTA-free protease inhibitors (Roche)) containing either 0.3% CHAPS or 1% Triton X-100. After centrifugation at 13,000Xg for 10 min, 4 μg of the indicated antibodies were added to the cleared supernatant and incubated with rotation for 90-min. 20 μl of a 50% slurry of protein G-sepharose was then added and the incubation continued for 1 h. Captured immunoprecipitates were washed four times with Lysis Buffer and once with Wash Buffer (50 mM Hepes pH 7.5, 40 mM NaCl, and 2 mM EDTA). Samples were resolved by SDS-PAGE and proteins transferred to PVDF and visualized by immunoblotting as described (Kim et al., 2002, Cell, 110:163-175). In vitro mTOR kinase assays were also as described (Kim et al., 2002, Cell, 110:163-175).

Two million HeLa cells in 6 cm dishes and transfected with the lentiviral plasmid encoding siRNAs targeting luciferase or mTOR were metabolically labeled by the addition of 0.5 mCi of [^{32}P]-orthophosphate (NEN) for 2 hr. Cells were rinsed twice with cold PBS and lysed in 300 ul of ice-cold CHAPS Lysis Buffer. After centrifugation, the cleared supernatants were collected, rictor immunoprecipitates prepared as above using with the rictor antibody, immunoprecipitates washed four times in Lysis Buffer, and proteins resolved by SDS-PAGE and radioactivity incorporation visualized by autoradiography.

Plasmid and siRNA transfections

Effectene (Qiagen) was used to transfect 1.2 million HEK293T cells in 6-cm dishes with up to 1 μg of the expression plasmids indicated in the figure legends. 48 hours after DNA addition, the cells were rinsed once with PBS and lysed in 800 μl of ice-

cold Lysis Buffer containing either CHAPS or Triton X-100 and analyzed by immunoprecipitation and immunoblotting as above. Sequences and transfection conditions for synthetic siRNAs targeting lamin, mTOR, and raptor have been described (Kim et al., 2002, Cell, 110:163-175) and are available at http://web.wi.mit.edu/sabatini/pub/siRNA_sequences.html. The sequences of the sense and anti-sense strands of the siRNA targeting rictor are ACUUGUGAAGAAUCGUAUCdTdT and dTdTUGAACACUUCUUAGCAUAG, respectively. Those for PKCα are UCCUUGUCCAAGGAGGCUGdTdT and dTdTAGGAACAGGUUCCUCCGAC.

Lentiviral shRNA cloning, production, and infection

Desalted oligonucleotides (IDT) were cloned into LKO.1 (Stewart, et al., 2003, RNA, 9, 493-501) with the AgeI/EcoRI sites at the 3' end of the human U6 promoter. The sequences of the oligonucleotides are as follows:

(1) mTOR 609 sense:
CCGGTTCAGCGTCCCTACCTTCTTCTctcgagAGAAGAAGGTAGGGACGCTGATT TTTG.

(2) mTOR 609 antisense:
AATTCAAAAATCAGCGTCCCTACCTTCTTCTctcgagAGAAGAAGGTAGGGACG CTGAA.

(3) Raptor 4145 sense:
CCGGagggccctgctactcgcttttctcgagaaaagcgagtagcagggccctTTTTTG.

(4) Raptor 4145 antisense:
AATTCAAAAAagggccctgctactcgcttttctcgagaaaagcgagtagcagggccct.

(5) Rictor 3274 sense:
CCGGTACTTGTGAAGAATCGTATCTTctcgagAAGATACGATTCTTCACAAGTTT TTTG.

(6) Rictor 3274 antisense:
AATTCAAAAAACTTGTGAAGAATCGTATCTTctcgagAAGATACGATTCTTCACA AGTA.

The numbers described for the above oligonucleotides indicate the nucleotide positions in the transcripts (with position one set at the start codon) at which the 21 bp stem of the shRNA begins.

Plasmids were propagated in and purified from Stbl2 bacterial cells (Invitrogen) and co-transfected together with the Delta VPR CMV VSVG plasmids into actively growing HEK293T using Fugene (Roche) as described (Stewart, et al., 2003, RNA, 9, 493-501). Virus-containing supernatants were collected at 36 and 60 hours after transfection, and concentrated by ultracentrifugation for 1.5 hrs at 23,000 RPM in an SW28 rotor at 4 °C. Pellets were resuspended overnight at 4 °C in 1/600th of the original volume. Cells were infected twice in the presence of 6 μg/ml protamine sulfate, selected for puromycin resistance and analyzed on the 5th day after infection.

Generation and application of dsRNA for RNAi in Drosophila S2 cells

Primers were designed within the coding sequence of each respective gene to amplify a 700 – 800 bp cDNA fragment. The following primers were used:

(1) EGFP forward: ATGGTGAGCAAGGGCGAGGAGCTGT;
(2) EGFP reverse: TTACTTGTACAGCTCGTCCATGCCG;
(3) dTOR (CG5092) forward: CAGGAGTTATTTTAAATGTGCTTCG;
(4) dTOR reverse: CCAAAATTCTTTGATCAGCTTAAAA;
(5) dRaptor (CG4320) forward: TGTCTGACAACACCCATTAACATAG;
(6) dRaptor reverse: GTACTTGTATTCCTTGACCAGATCC;
(7) dRictor (CG8002) forward: GCTTATTCCTAGACAGCATTATCCA;
(8) dRictor reverse: TTTTGAGTACTTCGATGCCTTTTAC;
(9) dS6K (CG10539) forward: CCTTCATAGTGGAGCTAGTTTATGC;
(10) dS6K reverse: CTTAGCGTTGTATCATCAGGTGAAT.

Each primer included a GAA and T7 promoter sequence (GAATTAATACGAC TCACTATAGGGAGA) at its 5' end. Primers were used in a one-step RT PCR reaction (Qiagen) to amplify a cDNA fragment using total Drosophila S2 cell RNA as template. The total RT-PCR reaction was purified using a PCR purification column (Qiagen) in a

final volume of 40 µl. 8 µl of the RT-PCR product was then used as a template in a 20 µl in vitro transcription reaction using the Megascript kit (Ambion) to generate the corresponding dsRNA fragments. The GFP template was amplified from an EGFP expression plasmid (Stratagene). Drosophila S2 cells actively growing in Schneider medium (Life Technologies) were washed and resuspended in Drosophila SFM (Life Technologies) to a final density of $1X10^6$ cells in 1 ml volume. 30 µg of dsRNA was added to the 1 ml of cells in SFM and incubated for 45 min at 25 °C. 2 ml of Schneider medium with 10% serum was then added back to the cells. After 24 hours, the cells were starved again and an additional 30 µg of dsRNA was added. After 4 days cells were harvested, washed once with cold PBS, lysed in the 1% Triton X-100 Lysis Buffer and analyzed by immunoblotting as above. Antibodies developed against mammalian phospho-S6K1, phospho-PKCα and PKCα were used to detect the Drosophila homologues of these proteins/modifications.

Immunofluorescence

HeLa cells transduced with the siRNA-expressing lentiviruses or transfected with synthetic siRNAs were cultured overnight on fibronectin-coated glass coverslips. Cells were fixed for 15 min with 3.7% formaldehyde in phosphate buffered saline (PBS), permeablized for 10 min in 0.5% NP40 in PBS containing 1 mM $CaCl_2$ and $MgCl_2$ (PBS+), and washed twice in PBS+. Nonspecific binding sites were blocked for 30 min by incubating in PBS+ containing 10% fetal bovine serum followed by 1 hr incubation in the same blocking buffer containing a 1:1000 dilution of the paxillin antibody. After washing in PBS+, blocking buffer containing 1:800 of the Alexa Fluor 488-conjugated secondary anti-mouse antibody, 1:800 of Texas Red-X-phalloidin and 1:1000 of Hoechst dye was added to the coverslips for 1 hr. The coverslips were then washed twice with PBS+, mounted in glycerol containing 0.1% p-phenylenediamine on glass slides, and visualized with fluorescence microscopy.

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.